CELL-MEDIATED AND HUMORAL IMMUNE RESPONSES TO *Dirofilaria immitis* IN EXPERIMENTALLY-INFECTED DOGS

By

Robert Burton Grieve

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate Council of
the University of Florida in Partial Fulfillment of the Requirements
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The canine immune response to *Dirofilaria immitis* was investigated in 3 aspects. Antigens from *D. immitis* adults and microfilariae were isolated and partially characterized and the humoral and cell-mediated immune responses to *D. immitis* were studied in experimentally-infected dogs.

Antigens from soluble somatic extracts of *D. immitis* males, females, and microfilariae were separated using preparative isoelectric focusing. The fractions obtained by isoelectric focusing were characterized by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (PAGE-SDS) and by immunodiffusion against canine sera from *D. immitis*-infected dogs and homologous rabbit antisera. Adjacent fractions from each preparation were combined on the basis of isoelectric point, similarity of protein constituents, and antigenic identity. The combined fractions were further characterized by polyacrylamide gel electrophoresis (PAGE). Gels were stained with coomassie blue for protein constituents and periodic acid

Schiff's stain and alcian blue were used to detect carbohydrate moieties. Triton X-100 was used to solubilize cuticle-bound antigens from *D. immitis* males and females; the resultant preparations were characterized by the same scheme employed for the soluble somatic fractions.

Soluble somatic preparations of males, females, and microfilariae were divided into 7, 7, and 3 final fractions, respectively. Considerable complexity and a wide molecular weight range of constituent proteins were observed in most fractions. All male and female fractions and 2 of 3 microfilaria fractions reacted with canine sera from *D. immitis*-infected dogs and homologous rabbit antisera. In contrast, the Triton X-100 solubilized preparations were less complex and demonstrated weak reactivity against canine sera from *D. immitis*-infected dogs and rabbit antisera. Both stains for carbohydrate determinations after PAGE were unsuccessful with the soluble somatic fractions.

A trichloroacetic acid-soluble, column-purified *D. immitis* antigen was used in an indirect hemagglutination assay to determine anti-*D. immitis* antibody titers in experimentally-infected dogs. Antibody titers were determined in 4 dogs with a single *D. immitis* infection, 4 dogs with a second *D. immitis* infection administered after the maturation of the first infection, and in 4 uninfected dogs. Sera for antibody determinations were collected every 14 days beginning 4 weeks before infection until 64 weeks after infection. Anti-*D. immitis* antibody was first detected in infected dogs 4 weeks after infection. Titers were highest at week 32, 2 weeks after the appearance of microfilariae, and diminished to low levels thereafter in the single infection group.

Antibody levels in the double infection group diminished as in the single infection group, but were demonstrable throughout the study. Antibody titers were higher ($p<0.05$) in infected dogs, but there were no significant differences in antibody titers between single and double infection groups.

The cell-mediated immune response was investigated using peripheral blood lymphocyte transformation. Lymphocyte transformation could not be induced with *D. immitis* antigens and the response to the mitogens phytohemagglutinin P, pokeweed mitogen, and concanavalin A were significantly depressed ($p<0.0001$) in infected dogs. Efforts to quantitate thymus dependent lymphocytes (T-cells) using nonimmune T-cell rosette formation with human red blood cells revealed no differences in T-cell numbers in infected and noninfected dogs. All dogs (infected and noninfected) were immunized with a T-cell dependent antigen, sheep red blood cells (SRBC), to investigate possible differences in T-cell functions in infected and noninfected dogs. No differences between groups in anti-SRBC antibody levels or 2-mercaptoethanol labile anti-SRBC antibody levels could be demonstrated.

INTRODUCTION

The canine heartworm, *Dirofilaria immitis*, has been the subject of considerable research. A more complete understanding of this infection is important both from the standpoint of the severity of the disease it may cause and possible ways to control the growing incidence of the parasite (Otto, 1974a). Further, the parasite often serves as a model for human filariasis research and may offer insights into the control of those infections. Research on methods to control *D. immitis* has been substantial but has been directed mainly to chemoprophylaxis and chemotherapy. Although chemoprophylaxis is reliable, it is not totally effective (Grieve and Bradley, unpublished information) and can be costly and inconvenient. Treatment to kill adult *D. immitis* is hazardous because of the danger of drug toxicity and the potential damage of emboli from dead parasites associated with the arsenical compound currently employed (Carlisle *et al.*, 1974).

As an alternative to chemotherapy, research on biologically-derived antigens to be used as vaccines has been emphasized in recent years, but it has become evident that before effective immunologic methods for the prevention or diagnosis of such infections can be developed, the responses of the host to the parasite and the mechanisms by which the parasite survives in an otherwise immunologically competent, natural host must be elucidated. The canine immune response to *D. immitis* has been the topic of several investigations, but these studies have often relied upon noncontrolled, natural infections or limited animal

numbers. In the few reports of experiments that utilized experimental infections, crude, nonspecific antigens were used which may have limited the amount of information produced.

When the data reported from previous investigations on *D. immitis* immunity are considered, a number of questions become apparent. In an effort to answer some of the questions, three major objectives were developed for this dissertation. The three major objectives addressed in this research were:

1. Isolation and partial characterization of antigens of *D. immitis* males, females, and microfilariae.

Rationale: Since different life-stages are present in the dog during the course of infection, information on the actual antigenic constituents and antigenic relationships of different stages (adult males, adult females, and microfilariae) is central to understanding the host immune response. Information on the complexity of the antigenic constituents of these stages is prerequisite to further study of possible genus-specific or stage-specific antigens.

2. Elucidation of the humoral immune response in dogs following primary and secondary experimental infections with *D. immitis*.

Rationale: In all previous reports concerning the humoral response in experimentally-infected dogs a crude, nonspecific antigen was used (Pacheco, 1966; Weiner and Bradley, 1972). Since a highly specific and sensitive adult worm antigen has been reported (Mantovani and Kagan, 1967), it was considered advantageous to use that antigen to study the antibody response in experimentally-infected dogs.

3. Investigation of the cell-mediated immune response to *D. immitis* in experimentally-infected dogs using the *in vitro* lymphocyte transformation technique.

Rationale: Assays of the cell mediated immune response to *D. immitis* have been either biological (Mantovani and Kagan, 1967) or have measured the *in vitro* response to a *D. immitis* antigen in an unnatural host (Kobayakawa, 1975). Examination of the response of lymphocytes from *D. immitis*-infected dogs to *D. immitis* antigens may provide new information on the nature of the immune response and on possible mechanisms of parasite survival.

The experimental design of this investigation was intended to help gain new, fundamental information on the canine immune response to *D. immitis* infections under controlled conditions. The intent of this study was to help in better understanding the infection and to explore the possibilities of any practical applications of that response.

LITERATURE REVIEW

Dirofilaria immitis Leidy, 1856, the canine heartworm, is distributed throughout the world; it is especially prevalent in warm, coastal regions and notably rare in Africa (Levine, 1968). In the United States, the infection appears to be extending from the enzootic Atlantic and Gulf Coast regions to the north and west and occurs in highly enzootic rates in certain temperate areas (Otto, 1974a)

The natural habitat of adult *D. immitis* is in the right ventricle and adjacent vasculature of the dog, *Canis familiaris*. However, the parasite has also been recorded as recovered from vomitus, feces, the eye, brain, spinal cord, abdominal cavity, bronchioles, various arteries, and abcesses (Otto, 1974b). Wild canines and felines may also serve as definitive hosts (Otto, 1974b) and a number of naturally-infected abnormal hosts have been reported including the black bear (Johnson, 1975), beaver (Foil and Orihel, 1975), wolverine (Williams and Dade, 1976), harbor seal (Medway and Wieland, 1975), California sea lion (Forrester *et al.*, 1973), domestic horse (Klein and Stoddard, 1977), and human (Otto, 1974b).

Experimental infections have been used to characterize the pathogenesis of *D. immitis* infection in felines (Donahue, 1975). Adult worm recoveries from experimentally infected cats are low, and dead worms found in many cats indicate that the dog is a more suitable host. Macaques have been experimentally infected to study *D. immitis* infections of primates (Wong, 1974). Parasites reached maturity only in those

macaques that were chemically immunosuppressed; it was concluded that a period of 60-90 days of diminished immune responsiveness after infection is necessary for the worms to reach maturity in the heart.

Adult *D. immitis* live preferentially in the right ventricle and pulmonary arteries of dogs. Adult males are 120-200 mm long and 700-900 μ wide with a spirally coiled tail characteristic of most filarid males; females are 250-310 mm long and 1.0-1.3 mm wide (Levine, 1968).

Microfilariae, the first-stage larvae, develop in the uterus of the adult female worm and are expelled without a sheath, gut, or genital primordium. The microfilariae are 286-340 μ long and 6.1-7.2 μ wide with a tapered anterior end and a straight tail (Lindsey, 1965). Microfilariae are present in the peripheral circulation of dogs with mature infections and have displayed seasonal (Kume, 1974) and daily periodicity (Pacheco, 1974). The mechanisms for periodicity are unknown (Masuya, 1976) but increased numbers of microfilariae in peripheral circulation during periods of greater vector availability facilitates perpetuation of the infection. A relatively constant number of microfilariae remain in the circulation of any given infected dog. The mechanism for maintaining a stable microfilariae population is unknown and withdrawal or addition of large numbers of microfilariae will not alter the population present in peripheral circulation (Wong, 1964).

Several mosquito species have been identified as proven *D. immitis* vectors (Levine, 1968). Microfilariae are ingested by the mosquito at the time of the blood meal and undergo two molts within the mosquito before they are infective as third-stage larvae. Third-stage larvae enter the dog where the mosquito has fed and molt in subcutaneous tissue to fourth-stage larvae in 9-12 days. A subsequent molt to fifth-stage

larvae occurs 60-70 days after infection (Orihel, 1961). A microfilaremia indicating maturity is usually evident at 6.5 months (Orihel, 1961).

D. immitis infections of dogs are typically diagnosed by demonstrating the characteristic microfilariae in the blood. However, no real correlation can be made between numbers of microfilariae and numbers of adult worms (Jackson, 1969; Pacheco, 1974). This method of diagnosis is further complicated by situations where adult worms are present without circulating microfilariae. Single sex infections or infections in an animal that will respond in a unique immunological fashion to microfilariae have no circulating microfilariae (Wong *et al.*, 1973).

Serious disease may result from a long-standing infection with *D. immitis*, especially if a large number of worms are present in the dog's circulatory system. Adult worms may produce a chronic endocarditis and dilation of the right heart; lungs may be congested and are typically effected by the granulomatous responses to thrombi formed from dead worms (Levine, 1968). Perhaps the most important pathology in the lungs is the resultant endarteritis and obstructive fibrosis (Adcock, 1961). In chronic cases, the liver may become enlarged and ascites will ensue. A terminal liver failure syndrome has been described in experimentally-infected dogs (Sawyer and Weinstein, 1963). Glomerular changes have also been reported in infected dogs. Although an immune complex associated etiology has been postulated (Casey *et al.*, 1972), evidence indicates that lesions are related to damage caused by motile microfilariae (Klei *et al.*, 1974; Simpson *et al.*, 1974).

Most reports indicate that there is not a protective immune response to *D. immitis*. Adult heartworms are patent for at least two

years (Levine, 1968) and microfilariae may persist when transfused into uninfected dogs for as long as two years (Underwood and Harwood, 1939). Under natural conditions in enzootic areas, dogs may be repeatedly infected over a lifetime, but after subjective analysis of such reports, it appears that the infection levels are not additive.

There is experimental evidence that dogs can be successfully immunized against *D. immitis* with irradiated *D. immitis* infective larvae (Wong *et al.*, 1974). Dogs that received varying numbers of gamma-irradiated third-stage larvae injected on different schedules revealed up to 0% recovery of adult worms after challenge with nonirradiated larvae. Antibody levels were not high and there was no anamnestic response after repeated challenge with either irradiated or nonirradiated larvae.

Since *D. immitis* is closely related to many of the human filarids it is often used as a source of antigen in the immunologic diagnosis of human filarial infections. Cross-reacting antigens among several filarids have been demonstrated (Neppert, 1974) and the availability of *D. immitis* makes it a good antigen source. Early antigenic preparations for diagnostic use were typically overnight saline extracts of macerated microfilariae (Franks and Stoll, 1945) or adults (Kagan, 1963). Presently, saline extracts of lyophilized adult *D. immitis* are used to obtain antigen preparations for the serodiagnosis of human filariases at the National Communicable Disease Center (Kagan and Norman, 1976). Indirect hemagglutination and bentonite flocculation tests employing this antigen have been relatively sensitive, but cross-reactivity is not restricted to filarids. Unfortunately, cross-reactions with the sera of patients infected with other nematodes, cestodes, trematodes,

protozoans, and even bacteria have been reported (Kagan and Norman, 1976). Pacheco (1966) reported on the sensitivity and specificity of different *D. immitis* whole worm extracts. In this study, a saline extract of lyophilized worms, a delipidized saline extract of lyophilized worms, acid-insoluble and acid-soluble protein extracts, and an ethanol extract were compared. The acid-insoluble fraction and the ethanol extract did not react with known positive anti-*D. immitis* canine antisera using indirect hemagglutination. The acid-soluble protein preparation was the most specific and sensitive of the antigen preparations tested, but sera of some animals infected with other helminths often cross-reacted.

Fluorescent antibody techniques have also been used with crude antigen preparations of adults (Ellsworth and Johnson, 1973; Wong, 1974), microfilariae (Wong, 1974; Qualls *et al.*, 1975), and third-stage larvae (Wong, 1974). Wong (1974) reported the ability to discern stage-specific antibody using an indirect fluorescent antibody technique with crude adult, microfilarial and larval antigens.

The initial, extensive purification procedures of *D. immitis* antigens were reported in 1965 (Sawada *et al.*, 1965). That work was designed to isolate filarial skin test antigen to diagnose human filarial infections. An aqueous, soluble somatic antigen preparation from adult *D. immitis* was treated with trichloroacetic acid and purified by gel filtration and ion-exchange chromatography. A fraction demonstrating a good degree of specificity and sensitivity was isolated and has been proven to be of benefit (Smith, 1971). In later work (Sawada *et al.*, 1970), anion-exchange chromatography, disc electrophoresis, and isoelectric focusing were employed to show at least 17 different proteins

in the isolated fraction. The various subfractions obtained were variably reactive in the skin test and were still relatively complex.

The isolation and purification scheme of Sawada *et al.* (1965) was repeated to obtain a highly specific and sensitive antigen fraction for use in the diagnosis of canine heartworm infection (Mantovani and Kagan, 1967). This antigen was reactive in skin tests and by the indirect hemagglutination assay it was shown to be specific for *D. immitis* when tested in dogs naturally infected with *D. immitis*, *D. repens*, and *Dipetalonema reconditum*. The advantage of the genus and species specificity of this antigen would be unique but, unfortunately, it has not been used in studies on the kinetics of the canine humoral immune response to *D. immitis*.

Another study reported that aqueous soluble extracts of *D. immitis* adults and microfilariae were subjected to disc electrophoresis (Wheeling and Hutchison, 1971). This technique revealed at least 27 and 17 protein bands, respectively. Immunoelectrophoresis of the adult extract against homologous rabbit antisera showed 10 precipitin arcs; 2 arcs were noted against sera from a patient with suspected filariasis. One arc developed when the microfilariae extract was subjected to immunoelectrophoresis against the human sera.

Takahashi and Sato (1976) used a defatted aqueous-soluble extract of adult *D. immitis* for fractionation. A fraction that was very reactive and specific in serodiagnosis of *Wuchereria bancrofti*-infected patients was reported after gel filtration, anion-exchange chromatography, and ammonium sulfate precipitation. Disc electrophoresis showed at least five protein bands with activity from the purified fraction.

More recently countercurrent immunoelectrophoresis of *D. immitis* male and microfilarial aqueous soluble somatic antigens against homologous rabbit antisera was used to demonstrate apparent stage-specific antigens (Desowitz and Una, 1976). Additionally, this same technique was used with sera from hypermicrofilaremic dogs and rabbit anti-*D. immitis* antisera to demonstrate circulating soluble *D. immitis* antigens. Sera from humans with low microfilaremias or occult infections showed intense precipitin lines to microfilaria-specific antigens.

The humoral immune responses to experimental *D. immitis* infections in dogs were studied in long term experiments with experimental infections by 2 investigators (Pacheco, 1966; Weiner and Bradley, 1972). In one study, anti-*D. immitis* antibodies were detected by indirect hemagglutination as early as two weeks after experimental infection, but could not be detected by either indirect hemagglutination or complement fixation beyong the ninth month postinfection (Pacheco, 1966). Peaks of antibody titers were observed at different times over the course of the prepatent and patent periods. It was postulated that they may have been related to antigenic changes in the parasite or to different antibody class responses. The author suggested that the lack of detectable antibody shortly after patency was due to absorption of most of the antibody by circulating microfilariae.

Weiner and Bradley (1972) investigated the humoral immune response after primary and secondary experimental *D. immitis* infections. A crude aqueous-soluble adult worm antigen in an indirect hemagglutination assay was used and serologic responses were similar to those reported by Pacheco (1966). A peak titer appeared about 22 weeks after infection and the amount of detectable antibody declined subsequent to the

appearance of microfilariae. Secondary infections administered after patency of the first infections did not produce an anamnestic response, but did delay the decrease in antibody titers as had been observed in the dogs infected only once.

In further work, Weiner and Bradley (1973) used single, radial immunodiffusion and 2-mercaptoethanol lability to demonstrate that immunoglobulin class M (IgM) was probably the primary active immunoglobulin throughout *D. immitis* infections. They speculated that immunoglobulin class G (IgG) may also have some role, but it was not demonstrable by the techniques used.

To date, most assays of the cell-mediated immune response have relied on skin testing. Mantovani and Kagan (1967) used the skin test to evaluate the column-purified antigen. The results obtained supported the serologic observations of *D. immitis* specificity.

Kobayakawa (1975) reported on extensive research on the guinea pig cell-mediated immune response to a defatted adult *D. immitis* extract. A response was confirmed by the migration inhibition test, the lymphocyte transformation test, skin test, and the skin reaction by passive transfer with sensitized peritoneal exudate cells. Cytotoxicity of peritoneal and splenic cells to microfilariae was demonstrated *in vivo* and *in vitro*. However, *in vitro* assays of the canine cell-mediated immune response to *D. immitis* have not been reported.

MATERIALS AND METHODS

Experimental Animals

Twelve pedigreed beagle dogs aged 9-10 weeks were obtained from a commercial supplier.[1] Immediately upon receipt the dogs were housed in a Rockefeller-type isolation building provided with temperature control and double-screened windows. Water and a commerically available feed[2] were provided *ad libitum*. Specific procedures were observed to maintain the rooms insect-free and the dogs helminth-free. The dogs were from 2 litters with 3 males and 3 females from each litter; throughout the experiment the animals were divided by sex. Rectal temperatures were determined daily and fecal analyses (Whitlock, 1948) were performed weekly on each dog for 12 weeks after arrival. Fecal analyses of all dogs were performed intermittently throughout the investigation to insure a helminth-free status. Previous to experimentation all dogs were subjected to a standard puppyhood vaccination regimen against canine distemper, canine infectious hepatitis,and leptospirosis.

Experimental Design

The dogs were divided into 3 groups of 4 animals each with 1 male and 1 female of each litter in each group. One group was an uninfected control group (Group A), the second (Group B) and third group (Group C)

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[1] Hazelton-Saunders, Inc., Midlothian, VA

[2] Gaines Meal, General Foods Corp., White Plains, NY

received single and double *D. immitis* infections, respectively.

Experimental Infections

The initial *D. immitis* infection was administered when the dogs were aged 10 months (Week 0). Approximately 500 female black-eye Liverpool *Aedes aegypti* that were fed on *D. immitis* microfilaremic canine blood were obtained from the College of Veterinary Medicine, University of Georgia, Athens.[1] Fifteen days after the infected blood meal the mosquitoes were dissected in Hank's balanced salt solution (HBSS)(pH 7.2). Infective larvae were individually counted into 1 ml disposable syringes;[2] after 30 larvae were counted into a syringe, it was filled to 1 ml with HBSS for inoculation. The larval inoculum was administered subcutaneously in the inguinal region of each dog in Groups B and C, then the syringes were filled with HBSS and the wash was inoculated subcutaneously, opposite the initial inoculation. Each syringe was carefully washed and the wash was examined microscopically for remaining larvae. Thirty-six weeks after the initial infection a second infection of the same larval number was administered to Group C.

Blood Collections

Blood was collected from the cephalic vein of each dog every 14 days beginning 4 weeks before experimental infection until 64 weeks after infection. Approximately 10 ml of blood was collected at each sampling; 2 ml were aspirated into a evacuated tube containing EDTA[3] and the

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[1] Provided by the U.S.-Japan Cooperative Medical Science Program (NIAID)

[2] Stylex^{R}, Pharmaseal Laboratories, Glendale, CA

[3] Vacutainer^{R}, Becton, Dickinson and Co., Rutherford, NJ

remainder was used for individual serum samples. Serum collected from whole blood was stored at -35°C for anti-*D. immitis* antibody determinations. Anticoagulant treated blood was used to count microfilariae (Weiner and Bradley, 1970) 2 and 4 weeks before infection, 2 weeks after infection, and then every 14 days beginning 22 weeks after infection.

Antigen Purification and Characterization

Protein Determinations

A standard curve was used to determine protein concentrations of all antigen preparations. A known quantity of bovine serum albumin[1] was serially diluted and the absorbance of each dilution was measured at 280 nm using a dual beam spectrophotometer.[2] This provided a standard curve so the absorbance of the various antigen preparations could be related to a protein concentration.

Crude Antigen Extraction

Adult *D. immitis* males and females were collected at necropsy from experimentally-infected dogs. The worms were separated by sex, washed three times in phosphate buffered saline (PBS)(pH 7.2), and frozen at -70°C. Microfilariae were collected by immersing female *D. immitis* recovered from naturally-infected dogs into PBS (pH 7.2) at 4°C for 2 hours; the adults were removed and the microfilariae were recovered after centrifuging[3] the PBS at 12,100 X g for 15 minutes at 4°C. Microfilariae were washed twice in PBS and frozen at -70°C.

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[1] American Monitor Corp., Indianapolis, IN

[2] Unicam SP1800 Ultraviolet Spectrophotometer, Philips Electronic Instruments, Mount Vernon, NY

[3] Beckman J-21C Centrifuge, Beckman Instruments, Inc., Palo Alto, CA

Each whole worm preparation was minced with a razor blade and mixed with approximately 5 times the worm volume of cold 0.01M 2-amino-2(hydroxymethyl)-1,3-propanediol(tris)(pH 8). The mixture was further disrupted using a french pressure cell press[1] at 2.0 X 10^4 pounds per square inch pressure. The homogenate was extracted overnight at 4°C on a continuous rocker[2] and then centrifuged at 12,100 X g at 4°C for 30 minutes; the supernatant was harvested and the precipitate resuspended in 10 volumes of 0.01M tris (pH 8) and further extracted for 72 hours. The combined supernatants served as the crude soluble somatic extract; the precipitate was extracted with PBS (pH 7.2) until protein was no longer evident and then subjected to detergent extraction.

Triton X-100[3] was used to solubilize any cuticle-associated proteins by the method of Harris *et al.* (1971). Only male and female preparations were in sufficient quantity for this extraction. The protein-extracted cuticular debris was lyophilized[4] overnight and added to 13% Triton X-100 (pH 7.5) at a rate of 0.1 g/ml and extracted by continuous agitation at 4°C for 72 hours. The mixture was centrifuged at 12,100 X g at 4°C for 30 minutes and proteins were obtained by adding 10 volumes of acetone to the supernatant at -35°C. The precipitate was collected after 24 hours, washed with ether, dried *in vacuo* and extracted for 24 hours with 0.01M tris (pH 8). The solubilized preparation was dialyzed

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[1] Aminco^R French Pressure Cell, American Instrument Co., Silver Springs, MD

[2] Lab-Tek^R Aliquot Mixer, Ames Lab-Tek, Inc., Westmont, IL

[3] Triton^R X-100, Fisher Scientific Co., Fair Lawn, NJ

[4] Labconco Freeze Dry-5, Labconco Corp., Kansas City, MO

against distilled water with 0.1% sucrose at 4°C for 48 hours and lyophilized. The Triton X-100 solubilized fractions of male and female cuticles are referred to as TSM and TSF, respectively.

Preparative Isoelectric Focusing

Approximately 100 mg of crude male soluble somatic antigen (MSSA), 120 mg of crude female soluble somatic antigen (FSSA), and 80 mg of crude microfilaria soluble somatic antigen (MFSSA) were individually subjected to preparative, flatbed isoelectric focusing.[1] The methodology observed is a modification of the techniques described by Winter *et al.*, (1975). Each crude preparation was dialyzed overnight against 1% glycine at 4°C. A slurry consisted of the aqueous antigen preparation, 5 g of Sephadex G-75 superfine,[2] 5.0 ml of ampholines pH 3.5-10[3], and distilled water to 100 ml was used to prepare each gel bed. The slurry was mixed, degassed and poured into a glass tray, then evaporated to reach 75% of the slurry crack-point. The gel bed was subjected to a continuous electric current at approximately 7 watts of constant power[4] at 10°C[5] for 14-16 hours. After focusing was complete, the current was discontinued and a dry filter paper (5 X 25 cm) was placed over the length of the gel bed for 2 minutes. The paper was removed and a fractionating grid was pressed through the gel bed. The filter paper was air dried, washed 3 times for 15 minutes each time in 10%

[1] LKB 2117 Multiphor, LKB Instruments, Inc., Rockville, MD

[2] Pharmacia Fine Chemicals, Pharmacia, Inc., Piscataway, NJ

[3] Ampholine^{R} pH 3.5-10 LKB Instruments, Inc., Rockville, MD

[4] Electrophoresis Power Supply, ISCO, Lincoln, NB

[5] Lauda K-4 R/D Brinkman Instruments, Messergate-Werk, Lauda, W. Germany

trichloroacetic acid (TCA), and stained with 0.2% coomassie brilliant blue[1] dissolved in methanol, water, and acetic acid (50:50:10). After staining was complete the paper was destained with methanol, water, and acetic acid in the same proportions until the background color was absent.

The pH of the gel bed at each of the 30 divisions created by the fractionating grid was determined;[2] this data and the staining patterns of the corresponding filter paper print were used to divide the gel bed into distinct fractions. When the constituent divisions of each fraction were determined, the gel bed within those divisions was harvested and washed into glass columns[3] (1 X 30 cm). The columns were eluted with distilled water and 5 ml volumes were collected until protein was not detectable. Total protein in each fraction was determined, then the fractions were dialyzed to remove ampholines, lyophilized, and solubilized with distilled water to a final protein concentration of 3 mg/ml. If less than 3 mg of protein was present the fraction was solubilized with 1 ml distilled water.

Production of Rabbit Antisera

Antisera for use in immunodiffusion was made in mature, female New Zealand white rabbits by intradermal inoculations (Vaitukaitus *et al.*, 1971) of crude soluble somatic or protein-extracted cuticle debris preparations. One mg of protein of male, female, and microfilarial crude

[1] Searle Diagnostic, High Wycombe, Bucks-England

[2] Corning Glass Works, Corning, NY

[3] Calbiochem, La Jolla, CA

soluble somatic antigens and 1 mg dryweight of male and female cuticular debris were administered to individual rabbits in a suspension of 1 ml of 0.01M tris (pH 8) and 1 ml of complete Freund's adjuvant.[1] The suspension was homogenized by passing it through a 20 gauge micro-emulsifying needle,[2] then inoculated intradermally at 25-30 sites on the back of each animal. At a separate site each animal received 0.5 ml of a vaccine containing diptheria and tetanus toxoids and a pertussis immunogen.[3] Animals were reimmunized by the same procedure 8 days later using Freund's incomplete adjuvant; the vaccine was not used at reimmunization. Twenty-nine days after the second immunization each rabbit received 1 mg of homologous crude antigen by intravenous administration in a marginal ear vein. An adjuvant-control rabbit received the same regimen without crude antigen. Blood was collected from all rabbits by marginal ear-vein puncture beginning 9 days after secondary immunization and then every 3-4 days for approximately 7 weeks. Sera collected from individual rabbits was pooled.

Immunodiffusion

The pooled rabbit antisera and pooled canine sera from dogs naturally-infected with *D. immitis* were used in immunodiffusion assays to compare adjacent fractions from preparative isoelectric focusing. The immunoglobulin portion of each pool was isolated by an ammonium sulfate precipitation technique (Harboe and Ingild, 1973). Twenty-five g of ammonium sulfate was added to 100 ml of each serum pool and

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[1] Calbiochem, La Jolla, CA

[2] Bolab, Inc., Derry, NH

[3] Wyeth^{R}, Wyeth Laboratories, Inc., Marietta, PA

this mixture was incubated for 20 hours at 22°C. After centrifugation at 12,100 X g for 30 minutes the supernatant was discarded and the precipitate was washed twice in 1.75M ammonium sulfate. The washed precipitate was solubilized with distilled water and dialyzed for 24 hours against distilled water and 24 hours against PBS (pH 7.2). Adjacent fractions collected from isoelectric focusing were compared for antigenic activity and identity against the immunoglobulin preparations of homologous rabbit antisera and canine immune sera using a standard immunodiffusion technique (Anonymous, 1968). Agarose,[1] sodium chloride and distilled water were mixed in the proportions 1:1:98, and heated to 90°C in a boiling water bath. Twelve ml of the hot solution was poured onto a level glass plate (9 X 9 cm), then after 2 hours at 4°C holes were cut[2] in the solidified gel and 10 µl of each fraction described above was diffused against 10 µl of purified immunoglobulin for 72 hours. Nonprecipitating protein was washed from the agarose with 1% sodium chloride for 48 hours, then the plates were rinsed in distilled water for 6 hours and dried. Staining was with 0.1% napthyl blue-black[3] in methanol, glacial acetic acid, and water (45:10:45); destaining was in the same solvent. Adjacent fractions showing antigenic identity were combined. TSM and TSF were diffused against homologous rabbit antibody and canine antibody to determine antigenic activity and complexity.

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[1]Biorad Laboratories, Richmond, CA

[2]Gelman Instrument Co., Ann Arbor, MI

[3]Sigma Chemical Co., St. Louis, MO

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate

Fractions combined after immunodiffusion were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (PAGE-SDS) to determine the complexity of each fraction and the approximate molecular weight of the fraction constituents. The principle methodology observed was reported by Weber and Osborn (1969) with adaptations for slab PAGE-SDS (Easterday *et al.*, 1976). Fractions combined after immunodiffusion were dialyzed for 72 hours at 4°C against 0.001M tris (pH 8) and lyophilized. Five hundred µg dry weight of each fraction and 50 µg of each of 6 protein standards[1] were used. The standards included cytochrome C, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, and catalase. Gels were cast in a gel-casting tower[2] in glass cassettes[3] (8.2 X 0.27 X 8.2 cm) using either 7.5%, 10%, or 12% polyacrylamide. The acrylamide solution was mixed as described (Easterday *et al.*, 1976), poured into the casting tower and allowed to polymerize for 30 minutes. After polymerization the gel cassettes were stored in a humidity chamber at 4°C for 14-16 hours. Before electrophoresis all samples were treated with 20 µl of 0.01M tris with 1% SDS and 1% 2-mercaptoethanol (2-ME) and incubated for 15 minutes at 60°C. After incubation, 5 µl of a bromophenol blue-sucrose solution[4] was mixed with each sample; 10 samples were applied

[1]Mol-Ranger™, Pierce Chemical Co., Rockford, IL

[2]Gel Slab Casting Apparatus GSC-8, Pharmacia Fine Chemicals, Piscataway, NJ

[3]Gel Cassette Kit, Pharmacia Fine Chemicals, Piscataway, NJ

[4]Bio-phore™, Biorad Laboratories, Richmond, CA

to each cassette. Electrophoresis was performed in a tris-glycine-SDS buffer at 90V in a specially designed chamber[1] until the tracking dye moved to 1.5-2.0 cm from the bottom of the gel. When electrophoresis was complete the gels were removed from the glass cassettes, rinsed in distilled water and fixed in 25% isopropanol, 10% acetic acid for 48 hours. After fixation, the gels were stained for 48 hours in 0.02% coomassie blue in 7% acetic acid; gels were destained with 25% isopropanol, 10% acetic acid until background color disappeared. The distance from the origin to the solvent front was measured as was the leading edge of each standard band and each distinct band of the individual fractions. The distance moved (mobility) of each standard of known molecular weight was plotted to obtain a standard curve for determining molecular weights of the fraction constituents. A standard curve for each polyacrylamide percentage was determined. Adjacent fractions from each of the MSSA, FSSA,and MFSSA with apparent identical constituents were combined.

Polyacrylamide Gel Electrophoresis

Fractions combined after PAGE-SDS were further examined using PAGE electrophoresis in 10% polyacrylamide with no SDS. Gels were cast as described above. Five hundred µg of each sample was solubilized in a tris-boric acid-EDTA electrophoresis buffer and 6 samples were applied to each cassette. Electrophoresis was performed at 120V until the solvent front was 1.5-2.0 cm from the bottom of the gel, then the gels were removed and split into halves with a wire cutter and gel-slicing

[1]Electrophoresis Apparatus GE-4, Pharmacia Fine Chemicals, Piscataway, NJ

frame.[1] Half of each gel was fixed in 20% sulfosalicylic acid for 40 minutes then stained for protein constituents with coomassie blue as before. The other half of each gel was used to stain for presence of carbohydrates. Initially, a periodic acid Schiff's stain (PAS) was used (Maurer, 1971). These gels were fixed in a sodium periodate, glacial acetic acid, hydrochloric acid and TCA solution for 16 hours, then washed for 8 hours in a glacial acetic acid, TCA solution and stained in Schiff's reagent[2] for 16 hours. When the PAS stain was complete, the same gel halves were stained with alcian blue[3] in a 0.5% solution in 3% acetic acid for 48 hours; destaining was with 3% acetic acid.

A general scheme for antigen purification and characterization is illustrated in Figure 1.

Humoral Immune Response Determinations

Purification of the Indirect Hemagglutination Antigen

Anti-*D. immitis* antibodies were measured in all sera collected using a semipurified antigen prepared according to Sawada *et al.*, (1965) and Mantovani and Kagan (1967). Adult *D. immitis* from naturally- and experimentally-infected dogs were separated by sex, washed 3 times in PBS, and extracted in 0.01M tris (pH 8) as described above. Equal protein quantities of each MSSA and FSSA were combined and 10% TCA was added until the pH reached 3.5. This mixture was left at 4°C

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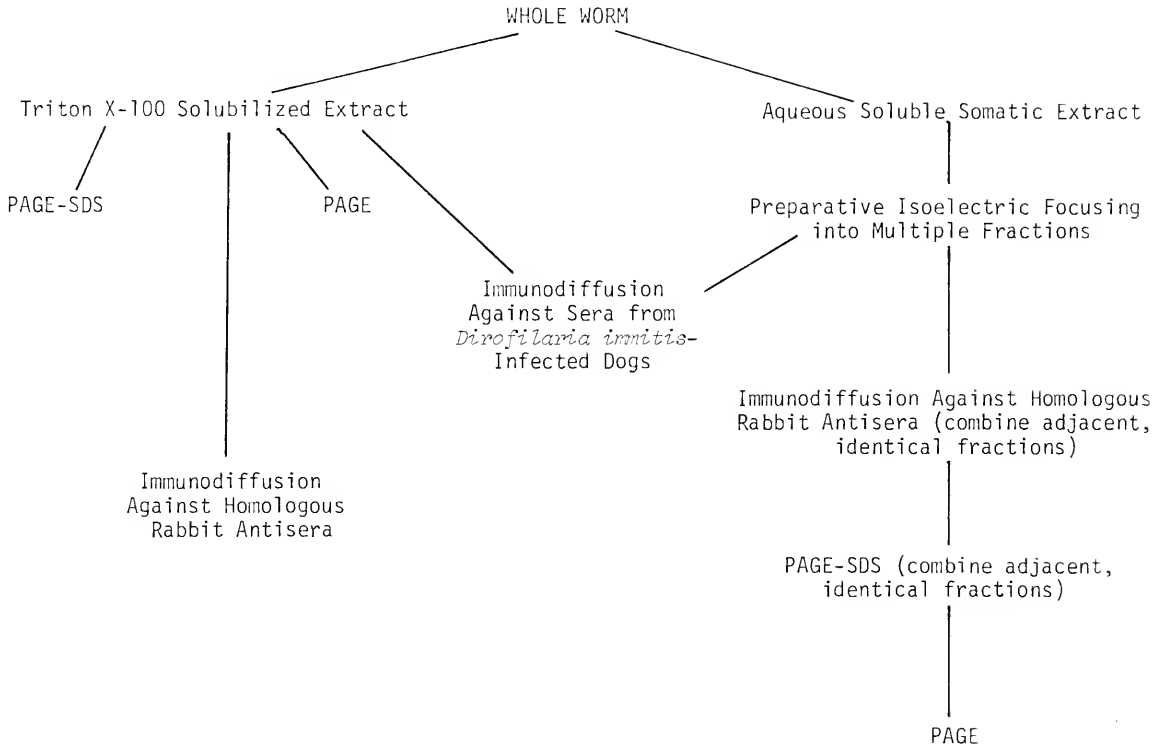
[1] Pharmacia Fine Chemicals, Piscataway, NJ

[2] Fisher Scientific Co., Fair Lawn, NJ

[3] Canalco, Inc., Rockville, MD

Figure 1. Diagrammatic representation of the scheme used in purification and characterization of *Dirofilaria immitis* antigens.


WHOLE WORM
Triton X-100 Solubilized Extract
Aqueous Soluble Somatic Extract
PAGE-SDS
PAGE
Preparative Isoelectric Focusing into Multiple Fractions
Immunodiffusion Against Sera from Dirofilaria immitis-Infected Dogs
Immunodiffusion Against Homologous Rabbit Antisera (combine adjacent, identical fractions)
Immunodiffusion Against Homologous Rabbit Antisera
PAGE-SDS (combine adjacent, identical fractions)
PAGE

for 1 hour, then centrifuged at 12,100 X g for 45 minutes at 4°C; the supernatant was dialyzed against distilled water for 48 hours at 4°C. Approximately 120 mg of TCA soluble protein was lyophilized and reconstituted to 20 ml with distilled water. Group separation on this preparation was at 4°C, in a glass chromatography column (2.5 X 60 cm)[1] using Sephadex G-100[2] equilibrated with distilled water. Elution was with distilled water at a flow rate of 3 ml/hour. The eluate was continuously monitored[3] for protein at 254 nm and each 5 ml was collected using an automated fraction collector.[4] Those fractions within the initial, principal protein peak were combined, lyophilized and resolubilized with 5 ml of 0.005M sodium acetate (pH 4.6).

The sodium acetate solubilized preparation was layered onto a carboxymethyl cellulose[5] column equilibrated with sodium acetate (pH 4.6) and eluted at a flow rate of 20 ml/hour. The eluate was monitored and collected as above and after each protein fraction was completely eluted, the column was eluted with a buffer of increasing molarity or pH. The eluants used in succession were 0.005M sodium acetate (pH 4.6), 0.05M phosphate buffer (pH 6), 0.1M sodium chloride (pH 7), 0.2M sodium chloride (pH 7), 0.4M sodium chloride (pH 7), 0.1N

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[1] ISCO Chromatographic Column, ISCO, Lincoln, NB

[2] Sephadex G-100, Pharmacia Fine Chemicals, Piscataway, NJ

[3] UA-5 Absorbance Monitor, ISCO, Lincoln, NB

[4] Model 328 Fraction Collector, ISCO, Lincoln, NB

[5] CM-cellulose, Pharmacia Fine Chemicals, Piscataway, NJ

sodium hydroxide (pH 7). The fractions eluted with each of these eluants were collected separately; previous to use in the indirect hemagglutination (IHA) assay the fractions eluted with 0.2M and 0.4M sodium chloride were combined and dialyzed against PBS (pH 7.2) for 48 hours at 4°C. The protein concentration of this preparation was determined previous to antigen titration. A scheme for the purification of the IHA antigen is illustrated in Figure 2.

Indirect Hemagglutination Assay

The IHA method was a modification of a widely used technique in the serodiagnosis of parasitic diseases (Kagan and Norman, 1976). Approximately 5 ml of sheep red blood cells (SRBC) suspended in Alsever's solution[1] were washed 3 times in hemagglutination buffer (HAB)[2] (pH 7.3). Centrifugation was for 10 minutes each time at 800 X g.[3] After the final wash, the packed cells were adjusted to a 2.5% suspension in HAB and mixed with an equal volume of $1:2 \times 10^4$ tannic acid solution. This mixture was incubated at 37°C for 10 minutes, then the cells were washed twice and resuspended to a 2.5% solution with HAB. The tanned cells were sensitized with one of five dilutions (1:4, 1:8, 1:16, 1:32, 1:64) of the column purified antigen by adding an equal volume of the antigen dilution to a 2.5% suspension of tanned SRBC; the mixture was incubated at 37°C for 15 minutes. The antigen-sensitized SRBC were washed twice and adjusted to a 1.5% suspension with HAB with 1% heat inactivated

[1]Becton, Dickinson and Co., Cockeysville, MD

[2]Bacto Hemagglutination Buffer, Difco Laboratories, Detroit, MI

[3]PR-2 Refrigerated Centrifuge, International Centrifuge Co., Needham Heights, MA

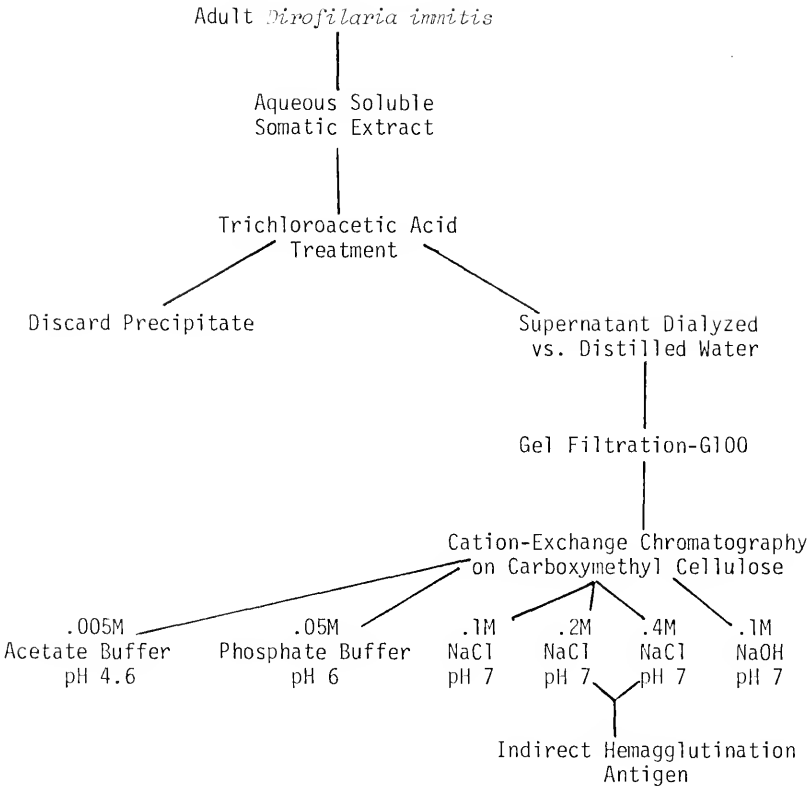

Figure 2. Diagrammatic representation of the procedure for purification of the *Dirofilaria immitis* indirect hemagglutination antigen

fetal calf serum (FCS).[1] Twenty-five µl of the diluent, HAB with 1% FCS, was added to each well of a V-bottom microtiter plate[2] in which serum dilutions were to be made. Fifty µl of either pooled canine immune serum or pooled canine serum from 4 dogs not infected (normal serum) were serially diluted with 25 µl dilution loops.[3] When the serum dilutions were complete, 25 µl of each antigen-sensitized SRBC suspension was added to each well in a dilution series of both normal and immune serum. The plates were incubated for 3 hours at 22°C; the highest serum dilution with hemagglutination was considered the titer. The antigen dilution giving the highest titer with the immune serum and no reaction with the normal serum was optimal; this dilution was used to determine anti-*D. immitis* antibody titers in individual serum samples collected over the course of the investigation. A diluent control was made by adding 25 µl of diluent and 25 µl of antigen-sensitized SRBC to 12 individual wells; a tanned SRBC control was made by adding 25 µl of unsensitized, tanned SRBC to each well of a dilution series of both normal and immune serum. In both controls it was necessary to obtain negative reactions. Differences in anti-*D. immitis* antibody titers of single infected, double infected, and noninfected dogs were statistically evaluated using a general linear models procedure (Steel and Torre, 1960).

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[1]Grand Island Biological Co., Grand Island, NY

[2]Cooke Laboratory Products, Alexandria, VA

[3]Beckman Instruments, Inc., Palo Alto, CA

Cell-Mediated Immune Response Determinations

Lymphocyte Isolation

The lymphocyte isolation technique was a modification of the method of Thilsted and Shifrine (1977). Fifteen ml of blood was obtained by venipuncture from the cephalic vein of each dog and mixed with heparin (10 U/ml of blood). The anticoagulant treated blood was mixed with 15 ml of RPMI-1640;[1] this mixture was divided in half, layered over 30 ml of a sucrose polymer-diatrizoate solution[2] with 0.1% methylcellulose. The tubes were centrifuged at 800 X g for 30 minutes at 22°C. The resultant lymphocyte-rich layer was harvested with a sterile pipette and washed in 50 ml of RPMI-1640. Total leuckocyte counts were made using a hemacytometer, then 200 µl of the preparation was fixed on a microscope slide with a cyto-centrifuge,[3] stained[4], and examined microscopically to obtain a differential leuckocyte count. Leukocytes were classified as either mononuclear or polymorphonuclear. After total mononuclear cell numbers were determined each cell preparation was washed in 50 ml of RPMI-1640 and resuspended to 3.0×10^6 mononuclear cells/ml in RPMI-1640 supplemented with 1% antibiotic-antimycotic[5] and 10% normal canine serum absorbed with human red blood cells (HRBC). This cell suspension was used for the lymphocyte-rosette assay, then adjusted to 1.25×10^6 mononuclear cells/ml for lymphocyte transformation.

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[1] Grand Island Biological Co., Grand Island, NY

[2] LSM™ Solution, Bionetics Laboratory Products, Kensington, MD

[3] Shandon-Elliott, Sewickley, PA

[4] Camco Quick Stain, Scientific Products, McGraw Park, IL

[5] Grand Island Biological Co., Grand Island, NY

Lymphocyte Transformation

Four major lymphocyte transformation experiments were performed 43, 45, 49, and 50 weeks after the initial *D. immitis* infection. All experiments were initiated after patency of the first infection and after Group C was infected the second time.

Two-tenths ml of each cell suspension was placed into each predetermined well in a microculture dish[1] to give 2.5 X 10^5 cells/well. Various levels of mitogen and antigen were assayed for lymphocyte transforming ability in quadruplicate cultures; unstimulated cultures from each cell preparation served as a background control. Phytohemagglutinin P[2] (PHA) was added in a range of 0.025-0.2 µl/culture, and Pokeweed Mitogen[3] (PWM) and Concanavalin A[4] (Con A) were added in ranges of 1-10 µl/culture and 1-10 µg/culture, respectively. The *D. immitis* antigen used was a combination of MSSA and FSSA (1:1) in PBS (pH 7.2) and was used in a range of 10-100 µg of protein/culture. All mitogens and antigens were solubilized in RPMI-1640 and were added to the respective cultures in a 10 µl volume. The cultures were incubated[5] for 48 hours at 37°C in 5% CO_2, then 0.5 µCi of tritiated thymidine[6] in RPMI-1640 was added to each well and incubation was continued under the same conditions for 16-20 hours. When the incubation

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[1]Cooke Laboratory Products, Alexandria, VA

[2]Difco Laboratories, Detroit, MI

[3] Grand Island Biological Co., Grand Island, NY

[4]Miles Laboratories, Inc., Elkhardt, IN

[5]Model CO-20, New Brunswick Scientific, New Brunswick, NJ

[6]The Radiochemical Centre, Amersham, England

was complete, the cultures were collected individually on a paper strip using an automated culture harvester.[1] The strip was air-dried and each portion of the paper with the cells from an individual culture was placed in a scintillation vial. The vials were filled with 4.5 ml of a scintillation cocktail;[2] after 3-6 hours of dark adaptation the radioactivity in each vial was measured using an automated liquid scintillation counter.[3] The amount of tritiated thymidine incorporated in cultures at each level of mitogen or antigen treatment was expressed as the mean counts per minute (cpm) of the 4 replications at each level. A stimulation index (mean cpm of stimulated culture/mean cpm of non-stimulated culture) was used to evaluate the extent of lymphocyte transformation.

Preliminary evaluation of lymphocyte transformation data indicated that there was no difference between dogs receiving single or double *D. immitis* infections; all data on cell-mediated immune response determinations are expressed as noninfected dogs (Group A) and infected dogs (Groups B + C). Further, because of limited animal numbers and the variability in lymphocyte responses it was necessary to evaluate the data after all 4 lymphocyte transformation experiments were combined. Additionally, data on the lymphocyte responses to all levels of each antigen and mitogen were combined to evaluate differences in lymphocyte responsiveness. Differences in mitogen and antigen responses were analyzed using a general linear models procedure.

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[1] Otto Hiller Co., Madison, WI

[2] Fisher Scientific, Pittsburg, PA

[3] LS 330, Beckman Instruments, Inc., Palo Alto, CA

Lymphocyte Rosette Assay

A modification of the procedure of Bowles *et al.* (1975) was used to quantitate canine lymphocytes forming nonimmune erythrocyte rosettes with HRBC. This assay was performed with the same cell preparations used in the 4 lymphocyte transformation experiments. Two-tenths ml of a 0.5% suspension of HRBC in RPMI-1640 and a 0.2 ml aliquot of each lymphocyte suspension at 3.0 X 10^6 cells/ml were mixed in triplicate. This mixture was incubated at 22°C for 30 minutes then centrifuged at 200 X g for 5 minutes. The cell pellet was incubated at 4°C for 14 hours then gently resuspended; one drop of the suspension was mixed with one drop of a dilute acridine orange[1] stain (Brostoff, 1974). The stained suspension was examined in a hemacytometer at 100X using fluorescent microscopy. One hundred fluorescing cells were counted and cells binding two or more erythrocytes were counted as rosette-forming cells. The percentage of rosette-forming cells in each cell suspension was expressed as the mean of the triplicate samples.

Immune Response to Sheep Red Blood Cells

After the initial data on the lymphocyte transformation experiments were obtained, it was apparent that a comparison of the immune response of infected and noninfected animals to a heterologous antigen, SRBC, would be advantageous. Three ml of a 20% SRBC suspension in PBS (pH 7.2) was administered intravenously to all dogs 49 weeks after the initial *D. immitis* infection and again 16 days later. Sera was collected on the day of the primary immunization and 8, 16, 20, 22, 27, and 29 days thereafter for anti-SRBC antibody determinations. Antibody was measured

[1]Eastman Kodak Co., Rochester, NY

by direct hemagglutination. Two 25 µl aliquots of each serum sample were mixed with either 25 µl of HAB with 1% FCS or 25 µl of 0.1M 2-ME and incubated at 37°C for 1 hour (Scott and Gershon, 1970). After incubation, the sera was diluted in a microtiter system as described above and 25 µl of a 1.5% solution of SRBC was added to each well. Titers for untreated and 2-ME treated sera were determined after incubation at 22°C for 3 hours. This assay was performed twice on each serum sample and the mean titer was recorded.

One hundred µl of sera from each dog 20 and 22 days after primary SRBC immunization was pooled and approximately 2 ml of this pool was separated by gel filtration on a Sepharacyl S-200[1] column (2.5 X 80 cm). The Sepharacyl S-200 was equilibrated and eluted with a sodium chloride, trizma base, hydrochloric acid buffer. The column flow rate was approximately 5 ml/hour and each 2.25 ml was collected in individual tubes on an automatic fraction collector.[2] Ultraviolet light absorbance at 280 nm of the eluate in each tube was determined and 4 pools corresponding to the manufacturer's predicted peaks for IgG, IgA, IgM and albumin fractions were collected. Each pool was concentrated with negative-pressure dialysis then assayed for 2-ME labile antibody as described above to insure that IgM was the only fraction demonstrating 2-ME lability. Differences in anti-SRBC antibody and 2-ME labile anti-SRBC antibody between infected and noninfected dogs were evaluated using a general linear models procedure.

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[1]Pharmacia Fine Chemicals, Piscataway, NJ

[2]Gilson Medical Electronics, Inc., Middleton, WI

RESULTS

Fecal examinations for helminth ova on all dogs were negative before and throughout the experimental period. All dogs remained healthy except for occasional minor lacerations obtained during fighting.

All dogs in Groups B and C received 29-30 infective larvae each. Dogs in Group C were infected with an additional 30 larvae each at the second *D. immitis* infection.

Microfilariae Counts

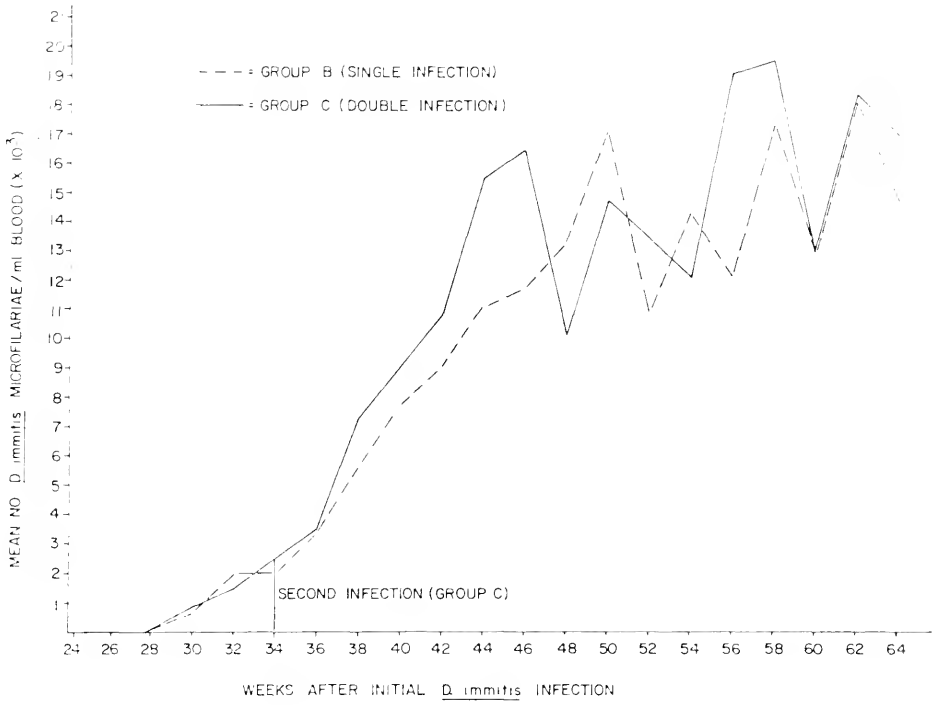
Microfilariae were first detected 26 weeks after infection in one dog and were present in all infected dogs by 30 weeks after infection (Appendix I). One dog that received 2 *D. immitis* infections became amicrofilaremic 56 weeks after the initial infection and 22 weeks after the second infection; microfilariae were not detected in that dog again throughout the study (Appendix I). A marker dog was not used to detect the onset of patency of the second infection; there was no evidence of a difference in microfilariae counts between single and double infected dogs at any time (Figure 3).

Antigen Purification and Characterization

Preparative Isoelectric Focusing

The preparative, flatbed isoelectric focusing effected a good protein separation with a reproducible pH gradient and approximately a 70% protein recovery of the crude soluble somatic preparations. MSSA, FSSA, and MFSSA were initially divided into 17, 22, and 9 fractions,

Figure 3. Mean numbers ($X10^3$) of *Dirofilaria immitis* microfilariae per ml of blood in Group B (single infection) and Group C (double infection).


- - - = GROUP B (SINGLE INFECTION)
——— = GROUP C (DOUBLE INFECTION)
MEAN NO D immitis MICROFILARIAE/ml BLOOD (x 10^3)
SECOND INFECTION (GROUP C)
WEEKS AFTER INITIAL D immitis INFECTION

respectively, based on the isoelectric points and the staining intensity of apparent protein bands on the filter paper print. The filter paper print of each separation, the percentage of total recovered protein in each final fraction, and the pH gradient established during separation are illustrated in Figures 4-6.

Immunodiffusion

After immunodiffusion of the fractions separated by isoelectric focusing, the male, female, and microfilaria fractions were combined to 11, 14, and 6 fractions, respectively. If antigenic identity could be established, adjacent fractions were combined. Antigenic activity against both homologous rabbit antibody and canine antibody was present in most fractions (Tables 1-3). Adult worm fractions with the most apparent antigenic activity and complexity were within an isoelectric point (pI) range of approximately 4.8-6.5.

Approximately 4 mg of TSF and 2 mg of TSM were recovered. There was some apparent antigenic activity when these were diffused against homologous rabbit antibody and canine antibody, but precipitin lines were weak and the degree of antigenic complexity could not be discerned.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate

Adjacent fractions of each preparation with apparently identical protein constituents after PAGE-SDS were combined after consideration of isoelectric point ranges and antigenic identity. The male, female, and microfilaria fractions were combined to 7, 7, and 3 fractions, respectively, the final fraction number for each preparation. The approximate molecular weights of the protein constituents in the soluble somatic fractions are listed in Tables 1-3. The TSM and TSF preparations were less complex. The TSM had 2 protein constituents of

Figure 4. Preparative isoelectric focusing separation and percentage of recovered protein in the final fractions from the *Dirofilaria immitis* male soluble somatic extract.

PERCENTAGE OF RECOVERED PROTEIN
pH
pH —•—
M-1
M-2
M-3
M-4
M-5
M-6
M-7

Figure 5. Preparative isoelectric focusing separation and percentage of recovered protein in the final fractions from the *Dirofilaria immitis* female soluble somatic extract.

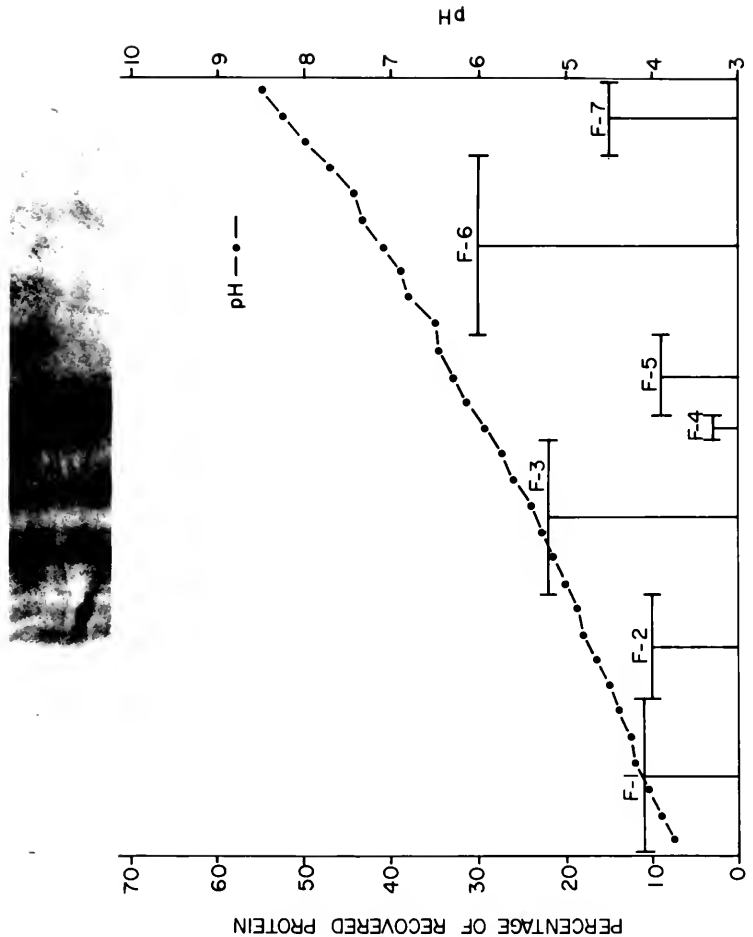

PERCENTAGE OF RECOVERED PROTEIN
pH
pH
F-1
F-2
F-3
F-4
F-5
F-6
F-7

Figure 6. Preparative isoelectric focusing separation and percentage of recovered protein in the final fractions from the *Dirofilaria immitis* microfilaria soluble somatic extract.

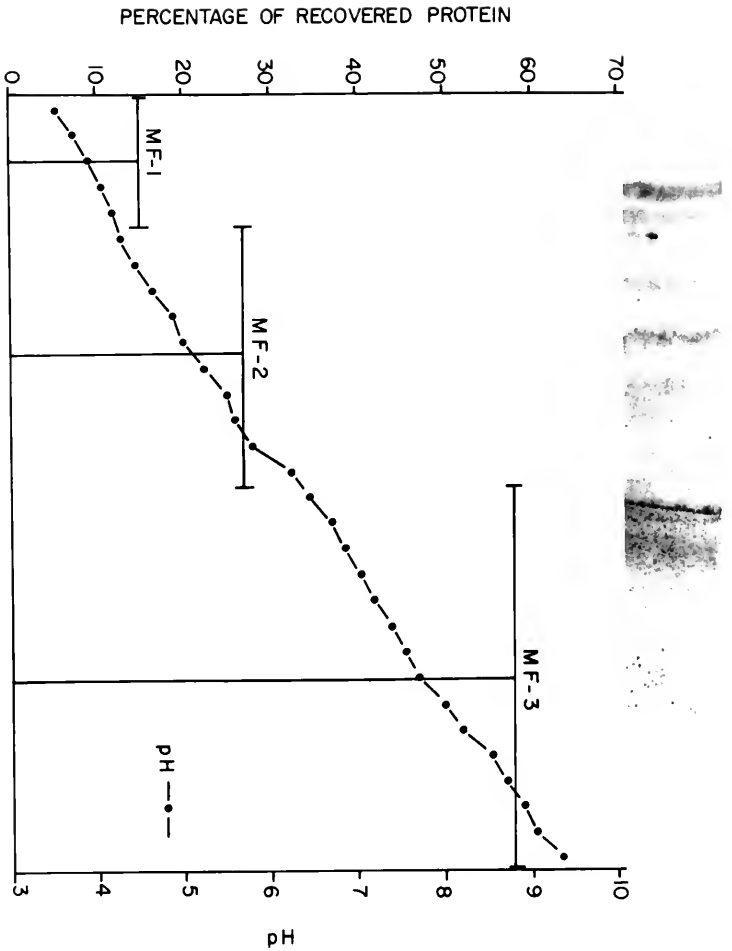

PERCENTAGE OF RECOVERED PROTEIN
0
10
20
30
40
50
60
70
MF-1
MF-2
MF-3
pH —•—
pH
3
4
5
6
7
8
9
10

TABLE 1. *Dirofilaria immitis* male soluble somatic antigen fractions purified by preparative isoelectric focusing and characterized by immunodiffusion, polyacrylamide gel electrophoresis, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Fraction	pI Range	Constituent Proteins after PAGE	Constituent Proteins after PAGE-SDS	Mol. Wt. of Constituent Proteins ($X10^4$)	Antigenic Activity[a]	
					Ra. anti-MSSA	Ca. anti-*D. immitis*
M-1	3.65-4.35	1	1	77	+	+
M-2	4.60-4.83	2	3	>100,56,18	+	+
M-3	4.93-5.35	3	7	90,68,56,52,43,30,18	+	+
M-4	5.43-6.10	4	9	68,56,52,43,36,30,26 24,17.5	+	+
M-5	6.20-6.40	2	6	68,43,30,26,20,17.5	+	+
M-6	6.57-7.80	1	4	68,30,26,20	+	+
M-7	8.23-8.50	1	7	>100,>100,>100,74 43,20,17.5	+	+

[a]Antigenic activity was determined by immunodiffusion.

TABLE 2. *Dirofilaria immitis* female soluble somatic antigen fractions purified by preparative isoelectric focusing and characterized by immunodiffusion, polyacrylamide gel electrophoresis, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Fraction	pI Range	Constituent Proteins after PAGE	Constituent Proteins after PAGE-SDS	Mol. Wt. of Constituent Proteins ($X10^3$)	Antigenic Activity[a]	
					Ra. anti-FSSA	Ca. anti-*D. immitis*
F-1	3.75-4.40	3	1	77	+	+
F-2	4.50-4.85	5	10	>100,82,77,59,42,36,33, 31,19,18	+	+
F-3	5.00-5.73	1	11	>100,85,77,68,59,42, 36,33,24,21,18	+	+
F-4	5.93	1	10	85,68,59,42,36,33 30,24,21,18	+	+
F-5	6.15-6.45	2	8	>100,77,68,42,33,24, 21,19	+	+
F-6	6.49-7.70	3	8	90,77,68,52,35,29,25,21	+	+
F-7	8.00-8.49	2	8	>100,>100,90,77,52 35,25,21	+	+

[a]Antigenic activity was determined by immunodiffusion.

TABLE 3. *Dirofilaria immitis* microfilaria soluble somatic antigen fractions purified by preparative isoelectric focusing and characterized by immunodiffusion, polyacrylamide gel electrophoresis, and polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Fractions	pI Range	Constituent Proteins after PAGE	Constituent Proteins after PAGE-SDS	Mol. Wt. of Constituent Proteins ($X10^4$)	Antigenic Activity[a]	
					Ra. anti-MFSSA	Ca. anti-*D. immitis*
MF-1	3.55-4.20	4	1	77	+	+
MF-2	4.30-6.25	2	1	77	+	+
MF-3	6.45-9.35	1	1	19	-	-

[a]Antigenic activity was determined by immunodiffusion

molecular weight 7.7 and 5.2 X 10^4; the TSF had 5 protein constituents of molecular weight 7.7, 7.1, 6.6, 5.2,and 3.4 X 10^4. Photographs of the separated fractions on polyacrylamide gel slabs appear in Appendix II. The standard lines for molecular weight determination obtained for each polyacrylamide gel percentage are in Appendices III-V.

Polyacrylamide Gel Electrophoresis

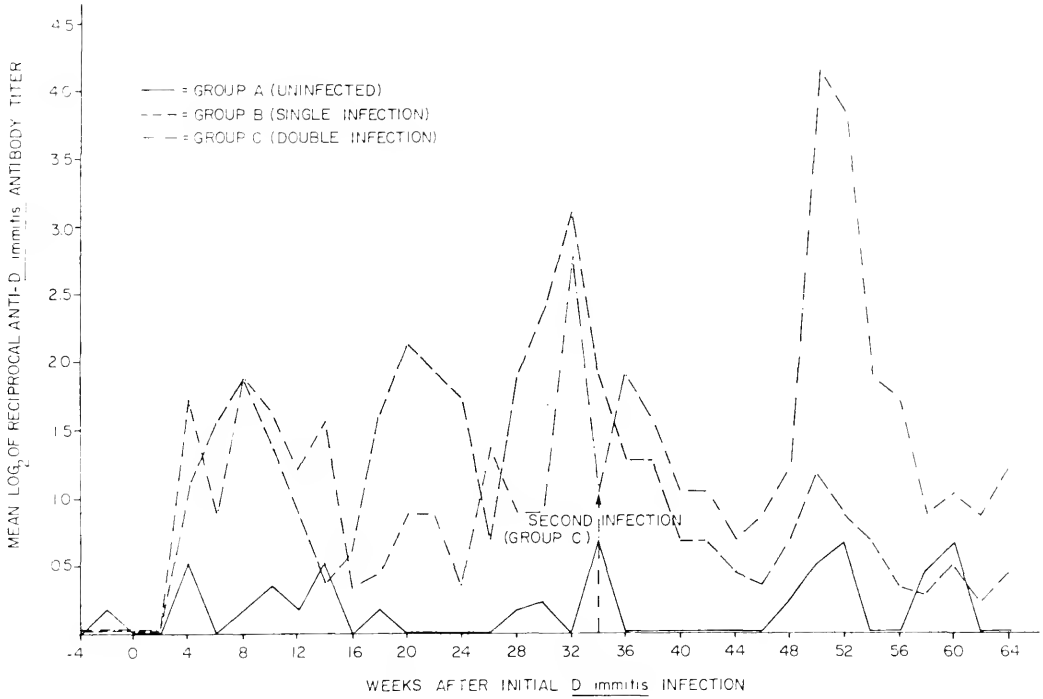
The number of proteins detected in individual fractions after PAGE are listed in Table 1-3. Two protein bands from the TSM preparation and 5 from TSF preparation were detected. Neither the PAS stain or the alcian blue stain were successful in detecting carbohydrate moieties associated with any of the separated protein constituents. The sample application point of all fraction and the electrophoresis tracks of the crude male and female preparations appeared to react with both stains, but staining was diffuse and could not be related to constituents of any of the fractions. Photographs of the PAGE separated soluble somatic fractions, crude soluble somatic preparations and Triton X-100 solubilized preparations are in Appendix VI.

Humoral Immune Response

Thirty mg of the column-purified antigen preparation was obtained from the initial 120 mg of TCA-soluble preparation. A dilution of 90 µg of protein/ml was optimal in sensitizing SRBC with antigen. A titer of 1:512 with pooled canine immune sera and no titer with pooled canine normal sera were repeatedly obtained with SRBC sensitized with this antigen dilution.

Anti-*D. immitis* antibody was first detected in Groups B and C 4 weeks after the initial *D. immitis* infection (Figure 7). Antibody

Figure 7. Mean $\log_2$ of the reciprocal anti-*Dirofilaria immitis* antibody titers in Groups A, B and C over the course of infection.


MEAN LOG2 OF RECIPROCAL ANTI-D immitis ANTIBODY TITER
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
—— = GROUP A (UNINFECTED)
– – – = GROUP B (SINGLE INFECTION)
– — = GROUP C (DOUBLE INFECTION)
SECOND INFECTION
(GROUP C)
-4
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
WEEKS AFTER INITIAL D immitis INFECTION

levels in the infected groups were significantly higher ($p<0.05$) than in the uninfected group. The antibody titer in Group B, the single infection group, began to decrease shortly after patency (Week 30) and antibody levels in Group C, the double infection group, persisted at low levels through Week 64. There were no significant differences between antibody titers of single and double infection groups after administration of the second infection. Individual anti-*D. immitis* antibody titers are tabulated in Appendix VII.

Cell-Mediated Immune Response

Lymphocyte Transformation

Peripheral blood lymphocyte transformation could not be induced in *D. immitis*-infected dogs with any level of *D. immitis* antigen tested (Table 4). A significantly ($p<0.0001$) depressed reactivity to PHA, PWM, and Con A was observed in lymphocyte cultures from infected dogs (Table 4).

Lymphocyte Rosettes

There were no differences evident in the mean percentage of rosette-forming cells between infected and noninfected dogs (Table 5). Although the standard deviation observed was low, and the mean percentage of rosettes remained reasonably consistent between experiments, the percentage of rosette-forming cells from the same dog often differed markedly between experiments.

Immune Response to Sheep Red Blood Cells

There were no significant differences in anti-SRBC antibody levels between infected and noninfected dogs (Figure 8). Although there appeared to be a greater quantity of anti-SRBC 2-ME labile antibody after the secondary infection (Figure 9), it was not statistically

TABLE 4. Mean counts per minute and stimulation indices from four canine peripheral blood lymphocyte transformation experiments of *Dirofilaria immitis*-infected dogs and noninfected dogs.

Culture Treatment	Group A (noninfected dogs)		Groups B + C (infected dogs)
none	84[a]		90
.025µl PHA	2152 (25.6)[b]		1263 (14.0)
.05µl PHA	4676 (55.7)	*	2588 (28.8)
.10µl PHA	7103 (84.6)		3740 (41.6)
.20µl PHA[c]	8607 (102.5)		4575 (50.8)
1µl PWM	3650 (43.5)		2129 (23.7)
5µl PWM	4164 (49.6)	*	2367 (26.3)
10µl PWM	3731 (44.4)		2132 (23.7)
1µg Con A[d]	5961 (70.9)		2741 (30.5)
5µg Con A	8501 (101.2)	*	5090 (56.6)
10µg Con A	9454 (112.6)		4329 (48.1)
10µg DIA[e]	96 (1.2)		90 (1.0)
50µg DIA	118 (1.4)		104 (1.2)
100µg DIA	103 (1.2)		100 (1.1)

*Statistically significant difference ($p<0.0001$)

[a]Mean counts per minute (cpm)

[b]Stimulation index (cpm of stimulated culture/cpm of nonstimulated culture

[c]This level was evaluated in one experiment

[d]This mitogen was evaluated in half of the dogs in each group in one experiment

[e]*Dirofilaria immitis* antigen

TABLE 5. Percentage of canine peripheral lymphocytes forming nonimmune rosettes with human erythrocytes.

Experiment	Group A (noninfected dogs)	Groups B + C (infected dogs)
1	24.7 ± 5.5[a]	24.6 ± 5.7
2	18.2 ± 1.8	33.2 ± 5.8
3	26.7 ± 5.2	31.6 ± 4.8
4	19.9 ± 5.5	28.8 ± 5.8

[a]Data is expressed as the mean leukocyte percentage of rosette-forming cells ± the standard deviation of triplicate samples from each dog.

Figure 8. Mean $\log_2$ of the anti-sheep red blood cell antibody titers in Group A and Groups B + C.

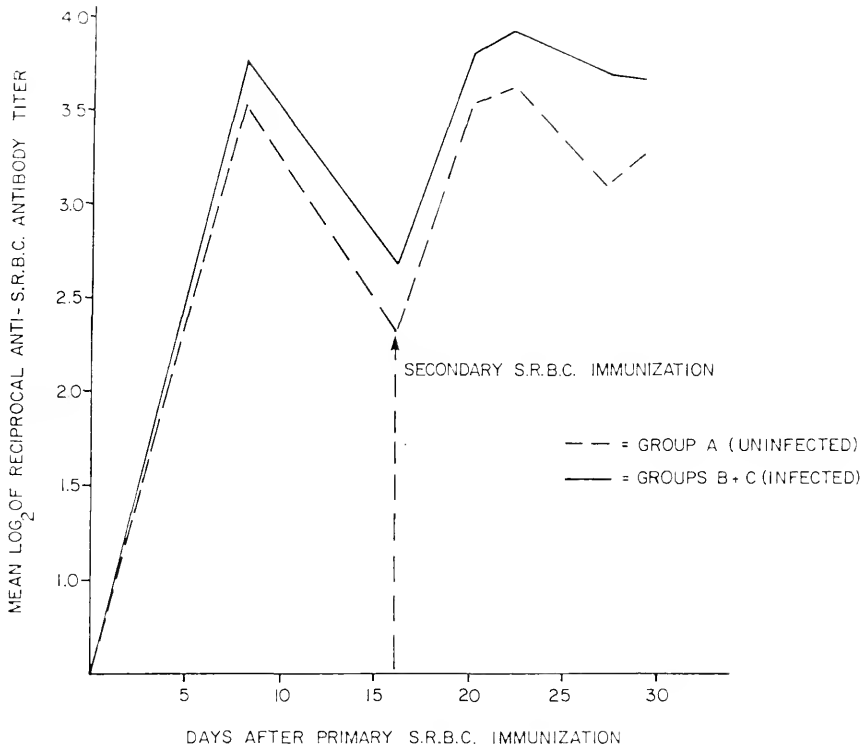
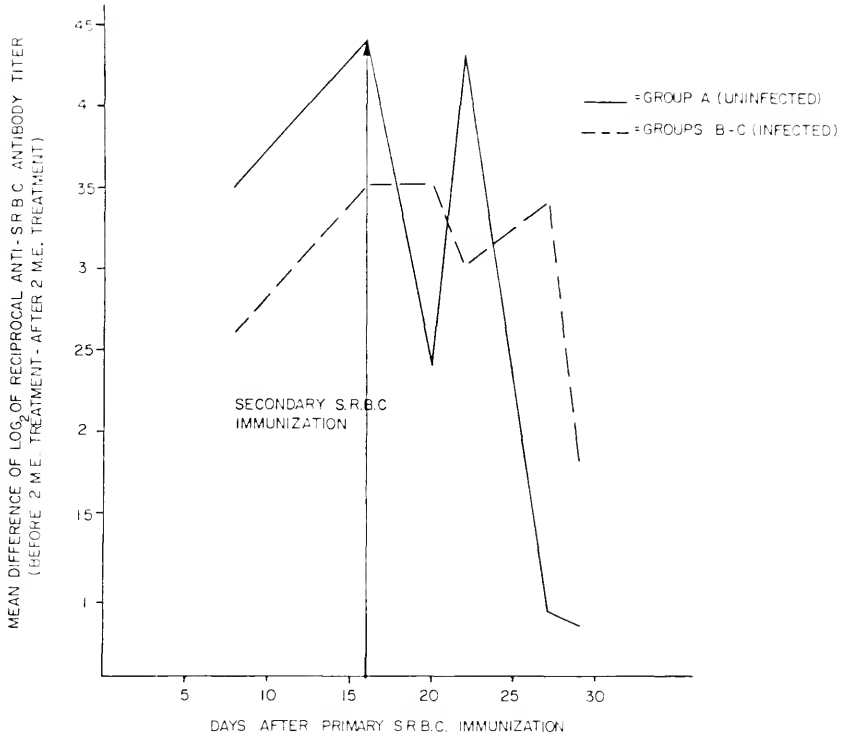
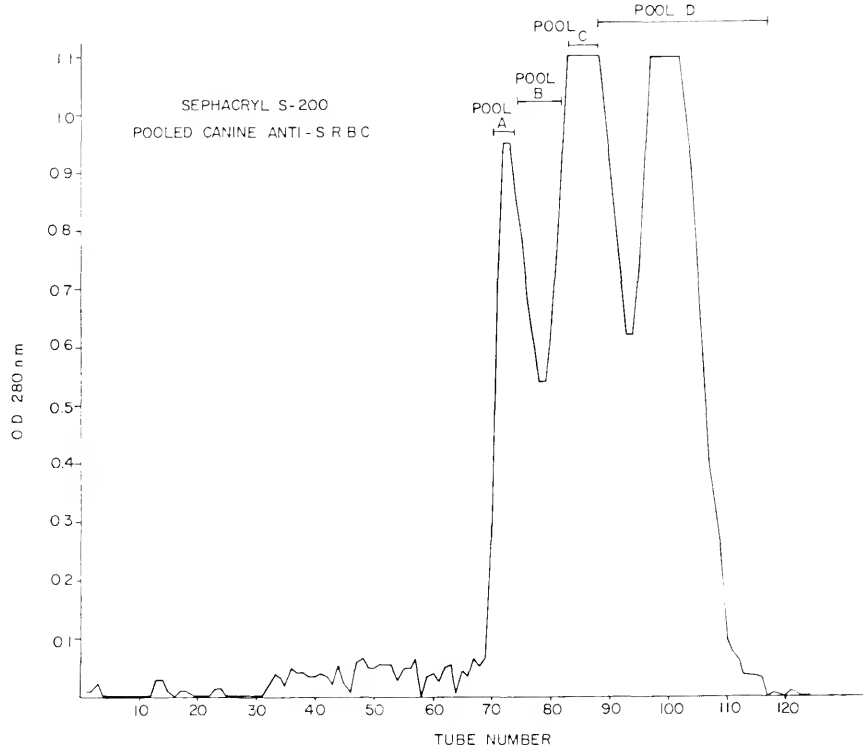

4.0
3.5
3.0
2.5
2.0
1.5
1.0
MEAN LOG2 OF RECIPROCAL ANTI-S.R.B.C. ANTIBODY TITER
SECONDARY S.R.B.C. IMMUNIZATION
— — = GROUP A (UNINFECTED)
—— = GROUPS B + C (INFECTED)
5
10
15
20
25
30
DAYS AFTER PRIMARY S.R.B.C. IMMUNIZATION

Figure 9. Mean difference of $\log_2$ of reciprocal anti-sheep red blood cell antibody titers before and after serum treatment with 2-mercaptoethanol.


MEAN DIFFERENCE OF LOG2 OF RECIPROCAL ANTI-S.R.B.C. ANTIBODY TITER
(BEFORE 2 M.E. TREATMENT - AFTER 2 M.E. TREATMENT)
4.5
4
3.5
3
2.5
2
1.5
1
5
10
15
20
25
30
DAYS AFTER PRIMARY S.R.B.C. IMMUNIZATION
SECONDARY S.R.B.C IMMUNIZATION
= GROUP A (UNINFECTED)
= GROUPS B-C (INFECTED)

significant. The average anti-SRBC antibody titers before and after 2-ME treatment for each dog are listed in Appendix VII. A good separation of the pooled anti-SRBC on Sepharacyl S-200 was effected (Figure 10). Anti-SRBC antibody activity was detected in Pools A, B,and C. Pool A and Pool C demonstrated the predominance of anti-SRBC activity. After 2-ME treatment the mean anti-SRBC antibody titer decreased from 1:8 to 1:1.5 in Pool A, the IgM pool. Antibody titers in Pool B, the IgA pool, and Pool C, the IgG pool, remained unchanged after 2-ME treatment.

Figure 10. Separation profile of pooled canine anti-sheep red blood cell serum on Sepharacyl S-200 and the pools collected for 2-mercaptoethanol lability determinations.


SEPHACRYL S-200
POOLED CANINE ANTI-S R B C
POOL A
POOL B
POOL C
POOL D
O.D. 280 n m
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
100
110
120
TUBE NUMBER

DISCUSSION

Antigen Purification and Characterization

Any definitive conclusions on the antigenic relationships of different *D. immitis* life-stages are not possible when the data from this investigation are evaluated; however, this study contributed to progress toward that end. The degree of antigenic complexity detected in the different life-stages is remarkable and with the partial-characterization data it will be helpful when further studies on antigenic relationships are considered.

Separating the myriad of proteins present in each crude soluble somatic extract with preparative isoelectric focusing proved to be advantageous. Recent reports have described disc electrophoresis, immunoelectrophoresis and countercurrent immunoelectrophoresis techniques that were successful in delineating the protein, and antigenic complexities of crude *D. immitis* extracts (Wheeling and Hutchison, 1971; Desowitz and Una, 1976). The principal deficit in the findings in both reports was that the separated and partially characterized fractions could not be directly related to immunological activity. In the present study, the fractions recovered after isoelectric focusing of large quantities of protein were easily harvested and could be used individually in immunological and biochemical characterization techniques.

There were antigens in all of the adult fractions and 2 of the 3 microfilaria fractions. The antigens were demonstrable using either

homologous rabbit antisera or canine immune sera; the predominance of antigenic activity and complexity in the adult worm fractions was in the pI range of approximately 4.8-6.5. In any further studies, the ampholines used in preparative isoelectric focusing could be confined to this pH range to concentrate on a more complete separation in this area. Since immunoelectrophoresis demonstrated at least 10 precipitin arcs in the crude adult *D. immitis* preparation tested against homologous rabbit antisera (Wheeling and Hutchison, 1971), further separation of the soluble somatic fractions will be necessary before antigenically distinct fractions are obtained.

PAGE-SDS was effective in determining approximate molecular weights as well as comparing the protein-staining constituents of adjacent fractions. Although there was some contamination evident in the proteins used in establishing the standard lines for molecular weight determinations, reliable lines could be established. The lowest molecular weight standard, cytochrome C, traveled into the solvent front in the 7.5% polyacrylamide resulting in a point inconsistent with the standard line (Appendix III). The 2 lowest molecular weight standards did not fit the standard line well in the 12% gels (Appendix V), but the line was consistent in the molecular weight range of most proteins present in the fractions. It is likely that the 12% polyacrylamide had too much cross-linking bis-acrylamide resulting in this effect (Weber and Osborn, 1969).

Stains for carbohydrate after PAGE were unsuccessful. Some diffuse staining with PAS and alcian blue was noted in the crude fractions; the staining appeared to be dispersed over the entire electrophoresis track

in each case. Additionally, there was some staining evident at the application point of each fraction. Wheeling and Hutchison (1971) reported 2 bands of carbohydrate-staining activity after disc electrophoresis of a crude adult extract; one band was near the origin and one was near the solvent front. It is possible that disc electrophoresis yielded sharper, more dense bands than the PAGE resulting in a more confined, intense stain.

It was unusual to note that in fraction F-1 and MF-1 more protein constituents were detected after PAGE than after PAGE-SDS. The 2-ME reduction before PAGE-SDS may have reduced large macromolecules to a number of small molecular weight proteins that traveled with the solvent front or different macromolecules may have had constituents of similar molecular weight that moved a common distance after reduction and PAGE-SDS.

The Triton X-100 solubilization of adult worm cuticles was largely unsuccessful. Unfortunately, only limited quantities of protein could be recovered. These preparations contained few proteins; relatively only a few bands appeared on PAGE and PAGE-SDS gels. Both preparations appeared to be weakly antigenic against both homologous rabbit antisera and canine immune sera when compared to soluble somatic preparations. Some success with Triton X-100 solubilization has been reported with *Onchocerca volvulus* antigens (Marcoullis and Grasbeck, 1976), but it was not clear if the worm preparations were thoroughly extracted in an aqueous system before Triton X-100 solubilization. It is possible that some of the antigenic complexity reported was due to residual aqueous soluble proteins.

Future work on the characterization of these soluble somatic antigen fractions should make use of immunologic techniques. After

having gained some information on the protein makeup, comparisons of the antigenic components of the fractions are necessary to learn about the possible stage- or sex-specific antigens. Antigenic comparisons of each fraction with every other fraction could be considered, but initially it would be important to compare those fractions from different preparations within the same isoelectric point range. Comparative immunodiffusion would be a logical technique to use in making the comparisons, but interpretation may be difficult. A radioimmunoassay technique has been successfully used in the determination of stage and species specificity of *Schistosoma mansoni* antigens (Hamburger *et al.*, 1976). This technique would be ideal for antigenic comparisons of the *D. immitis* fractions, but a greater degree of purity in the fractions will be necessary before the technique could be implemented.

Humoral Immune Response

Results obtained on antibody titers were largely comparable to data from other similar studies (Pacheco, 1966; Weiner and Bradley, 1972). Anti-*D. immitis* antibody was detectable between 2 and 4 weeks after infection and peak titers were reached 2 weeks after *D. immitis* reached patency in all dogs. It would be difficult to interpret, as Pacheco (1966) did, that microfilariae may be responsible for absorbing antibody and removing it from peripheral circulation, but the onset of the microfilaremia is obviously associated with the decrease in circulating antibody. Since the titers diminish 2-4 weeks after the appearance of microfilariae, the microfilariae may be directly affecting the immune system and the decline of antibody titers could be due to

the normal, biological half-life of the specific immunoglobulins. Weiner and Bradley (1972) reported that a second *D. immitis* infection did not result in a typical anamnestic response, although dogs infected a second time had prolonged antibody levels when compared to dogs with single infections.

The results illustrated in Figure 7 indicated that there was a delayed anamnestic response in the group receiving 2 infections, but that response was not statistically significant ($p>0.05$). On close observation, this apparent secondary response was related to unusually high antibody titers in the dog that became amicrofilaremic (Appendix VII). This animal developed high antibody titers at Week 50, just prior to the amicrofilaremic state and at the same point of the apparent anamnestic response. The unusual antibody response in this animal accounts for the artifact (Figure 7) when the antibody levels are illustrated; it also contributes to the enigma of the relationship of the microfilariae to the canine immune response during the progress of infection.

Mantovani and Kagan (1967) reported that the TCA soluble antigen used in the present investigation was both genus- and species-specific based on IHA and skin testing of naturally-infected dogs and Pacheco (1966) reported that an acid-soluble preparation of saline extracted adult *D. immitis* was highly specific in serologic testing of experimentally-infected dogs. It is impossible to assess the specificity of the purified, acid-soluble antigen preparation used without testing it against sera from dogs or other animals infected with other parasites, but evidence indicates that it is more specific than the crude preparations typically

used. The IHA titers for noninfected dogs reported by Weiner and Bradley (1972) were often as high as 1:256 with a crude saline-soluble antigen, making interpretations of antibody changes very difficult. On this basis alone, the purified antigen used in the present study was more specific; anti-*D. immitis* antibody titers of the noninfected animals never exceeded 1:2. Further, the antigen preparation used during this study may be more sensitive than crude preparations. Relative antibody titer increases were greater than titers reported after a similar infection schedule using a crude antigen preparation (Weiner and Bradley, 1972).

Based on these results, future work on the humoral immune response to *D. immitis* should be concentrated on a more specific and well-defined antigen and a better serologic test. As research progresses on the isolation and characterization of *D. immitis* antigens a stage-specific antigen may be obtained. Detection and quantitation of adult and microfilaria-specific antibodies over the course of infection may answer questions on the decrease in antibody after the appearance of microfilaria and on the nature of the antibody response preceeding the amicrofilaremic state in some dogs. The enzyme-linked immunoabsorbent assay has been used in serologic testing of *Onchocerca volvulus* (Bartlett *et al.*, 1975), another filarid parasite; if it is as sensitive and reproducible as reported for other systems (Voller *et al.*, 1976), it may be ideal for antibody determinations in *D. immitis* infections.

Cell-Mediated Immune Response

No evidence of *D. immitis* antigen-induced lymphocyte transformation of peripheral blood lymphocytes of infected dogs was obtained. In

addition to the antigens used in the 4 major lymphocyte transformation experiments, the acid-soluble IHA antigen and a crude microfilaria extract did not stimulate lymphocyte transformation in small-scale experiments. In contrast, successful lymphocyte transformation using similar antigen preparations has been reported in other filarial infections (Ottesen *et al.*, 1977; Portaro *et al.*, 1977). Ottesen *et al.* (1977) used antigens from saline extracted *D. immitis* and *Brugia malayi* adults to successfully transform peripheral blood lymphocytes from humans infected with *Wuchereria bancrofti*. Mean stimulation indices as high as 36 were recorded when *D. immitis* antigens were used to transform peripheral blood lymphocytes of noninfected, *W. bancrofti*-exposed patients. Portaro *et al.* (1977) have used saline extracts of *D. immitis*, *Trichnella spiralis* and *Brugia pahangi* to transform splenocytes from *B. pahangi* infected jirds. Each of the filarial antigen preparations induced lymphocyte transformation but the *T. spiralis* antigen did not. Of the 2 filarial antigens, the *B. pahangi* preparation was more effective in transformation. These data suggested a possible filaria-specific diagnostic test and confirmed that specific antigen-induced lymphocyte transformation was possible in a filarial system.

The failure to induce *D. immitis*-specific lymphocyte transformation in this study may have been related to a highly significant ($p<0.0001$) depression of mitogen responsiveness in infected dogs. Depressed mitogen responses have been reported in such diverse disease situations as cancers (Mannick *et al.*, 1977), leishmaniasis (Farah *et al.*, 1976), and malaria (Spira *et al.*, 1976). Several nonfilarial helminths have been associated with an immune supression phenomenon including *T. spiralis*

(Cypess *et al.*, 1973; Faubert and Tanner, 1975), *Nematospiroides dubius* (Shimp *et al.*, 1975), *Ascaris suum* (Crandall and Crandall, 1976), *Taenia crassiceps* (Good and Miller, 1976), and *Schistosoma mansoni* (Pelley *et al.*, 1976). Two diseases of dogs, demodectic mange (Scott, *et al.*, 1974 and 1976) and canine distemper (Krakowka *et al.*, 1975) have been associated with diminished mitogen responsiveness. Scott *et al.* (1974) reported a correlation between low mitogen responsiveness and *Demodex canis* infection and later work (Scott *et al.*, 1976) indicated that successful treatment for removal of the mite would reverse the phenomenon. Recently, various types of immune suppression have been documented for 3 other filarid parasites (Dalesandro and Klei, 1976; Ottesen *et al.*, 1977; Portaro *et al.*, 1976). Dalesandro and Klei (1976) using *Dipetalonema vitae* showed decreased antibody responses to bovine serum albumin in hamsters and jirds and to SRBC in hamsters. Immunizations at different times in the course of infection indicated that the onset of immunodepression was related to the appearance of microfilariae. Ottesen *et al.* (1977) claimed that a specific cellular unresponsiveness to *W. bancrofti* occurred in infected humans. Humans with infections, humans without infections but exposed to the parasite, and humans with no exposure to the parasite were immunized with tuberculin (PPD) and streptococcal (SK-SD) antigens. Lymphocyte responses to those antigens, filarial antigens and mitogens revealed that only the response to filarial antigens was impaired in infected humans; mitogen, PPD, and SK-SD responses were all normal. In other work, splenocytes from *B. pahangi*-infected jirds were shown to transform with exposure to filarial antigens, but mitogen responsiveness was

inversely correlated to the appearance of filarial-specific splenocytes (Portaro *et al.*, 1976).

It is possible that *D. immitis* antigen-induced lymphocyte transformation may have been observed if such experiments were attempted before patency; evidence (Dalesandro and Klei, 1976) would indicate that the immune suppression phenomenon may be associated with the microfilaremia. The decline in anti-*D. immitis* titers shortly after the onset of the microfilaremia would support the idea of a microfilaria-associated immune suppression. Further work should concentrate on the role of the microfilariae in this effect.

Since the 3 mitogens used in this study were either T-cell mitogens or T-cell dependent B-cell (thymus-independent lymphocyte) mitogens in other organisms, an impaired T-cell function or a decreased number of T-cells in the infected animals would explain the reduced responses. Enumeration of canine T-cells by nonimmune rosette formation with HRBC was reported (Bowles *et al.*, 1975) and this technique was implemented in this investigation. There were no differences in the percentage of rosette-forming cells between infected and noninfected dogs. Recent work (Krakowka and Guyot, 1977) demonstrated that canine eosinophiles formed nonimmune rosettes with HRBC. In the rosette procedure used in this investigation, acridine orange stain was used for ease of counting the cells. At a magnification of 100X leukocyte nuclear morphology was not easily determined thus a small percentage of the cells counted as lymphoid rosettes may have actually been eosinophilic leukocytes. Experiments were performed to examine the rosettes at high magnification and the results confirmed the work of Krakowka and Guyot (1977). The slightly

higher percentage of rosette-forming cells in infected dogs was probably due to more contaminating eosinophiles in the mononuclear cell preparation because of the chronic eosinophilia (Weiner and Bradley, 1972) in *D. immitis*-infected dogs.

In an attempt to assay T-cell function in *D. immitis*-infected dogs a heterologous T-cell dependent antigen, SRBC, was used to immunize the dogs and measure potential differences in antibody responses to that antigen. There were no differences between the groups in total hemagglutinating antibody or 2-ME labile hemagglutinating antibody. The 2-ME labile antibody responses seemed to occur primarily in the IgM class of immunoglobulin after secondary immunization of infected dogs and in the IgG class in noninfected dogs. This observation would support the hypothesis of impaired T-cell function, but it could not be substantiated with statistical analysis. Unfortunately, it was not possible to do antigen dose response studies of SRBC in the canine before immunization and the dose may have been too high; T-cell dependence or independence of the immune response to this antigen has been shown to be antigen concentration-dependent (Playfair and Purves, 1971; Lemmel *et al.*, 1971).

The implications of a state of diminished immune responsiveness in *D. immitis*-infected dogs are numerous. This phenomenon must be considered in evaluating the pathogenesis of *D. immitis* infections. It is reasonable to assume that this condition will predispose an infected dog to infectious and neoplastic diseases. Further, even though preliminary experiments on immunizing dogs with irradiated larvae have shown promise (Wong *et al.*, 1974), the results of the present investigation demonstrate that the complexity of the immune response to

D. immitis should be more clearly defined before it can be effectively manipulated to prevent infections.

One of the most interesting considerations in view of the depressed immune response is the possibility of treating the infection with an agent that will potentiate the immune response in conjunction with, or beyond, killing the parasite. Levamisole, a compound often used in immunologic enhancement (Bruley-Rosset, 1976), has been highly effective in killing *D. immitis* microfilariae (Bradley, 1976) and variably effective against adult worms (Boring and Shepard, 1974). The mode of action of levamisole has not been ascertained, but may be related to an enhanced immune responsiveness. This principle has been investigated in the treatment of human filariases (Pinon *et al.*, 1974), but inconclusive results were obtained. Experiments evaluating the effectiveness of an immune-enhancing agent against *D. immitis* in dogs may provide information to aid the treatment of human filariases as well as canine dirofilariasis.

CONCLUSIONS

Soluble somatic extracts of *D. immitis* males, females, and microfilariae were separated into 7, 7, and 3 fractions, respectively. The isoelectric point range, the number of protein constituents after PAGE and PAGE-SDS and the estimated molecular weight of each constituent after PAGE-SDS were determined. A good deal of complexity was present in most fractions as evidenced by specific protein staining of the polyacrylamide gels; stains to detect carbohydrate moieties were unsuccessful. Antigenic activity was demonstrated in each adult *D. immitis* fraction and 2 of 3 microfilarial fractions. Triton X-100 solubilization of adult *D. immitis* cuticles yielded preparations that were weakly antigenic with few protein constituents.

A TCA-soluble column-purified antigen was demonstrated to be more effective in the IHA test than previously used antigens. Antibody titers in experimentally-infected dogs decreased after the appearance of microfilariae. Titers from dogs infected only once dropped and remained at low levels following the appearance of microfilariae, but antibody levels in dogs infected a second time were demonstrable throughout the study.

D. immitis antigen induced lymphocyte transformation could not be effected in experimentally-infected dogs. This finding may have been related to a significantly ($p<0.0001$) depressed mitogen reactivity of peripheral blood lymphocytes of *D. immitis*-infected dogs. Quantitation of nonimmune T-cell rosettes and immunization of dogs with SRBC to assay

T-cell numbers and T-cell function, respectively, did not reveal any differences in infected and noninfected dogs.

APPENDICES

APPENDIX I

Dirofilaria immitis Microfilariae Counts Over the Experimental Period

Weeks After Initial *D. immitis* Infection	Group B (single infection)				Group C (double infection)			
	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
-4	0	0	0	0	0	0	0	0
-2	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	1	0
28	32	2	0	100	100	1	400	0
30	1100	500	1	900	1900	700	800	11
32	3500	500	100	3900	2200	2300	900	500
34	2500	900	300	4300	3100	5300	1200	200
36	4400	1300	1300	5100	5100	7000	1600	700
38	7100	2400	2900	10,200	8900	13,000	2600	4600
40	6400	2300	5000	16,900	9600	20,000	2400	4000
42	9000	4100	2800	20,700	24,400	11,900	4100	3000

Microfilariae Counts (Continued)

Weeks After Initial *D. immitis* Infection	Group B (single infection)				Group C (double infection)			
	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
44	11,800	3400	5800	23,400	19,400	29,500	3000	9800
46	12,600	6100	4200	23,800	27,000	30,200	5600	2900
48	12,500	4200	7500	30,900	24,900	5900	4600	4800
50	24,000	5600	7600	31,500	34,200	3600	7600	13,100
52	13,300	6400	2900	20,800	26,300	3700	7800	9800
54	15,200	7300	6800	27,800	28,600	4000	7200	8300
56	16,500	3700	5900	22,600	37,900	0	4900	14,300
58	20,600	7500	4800	36,300	29,400	0	7100	22,100
60	16,300	3700	5000	26,800	26,000	0	6400	9600
62	15,200	8200	9600	39,600	30,400	0	8700	15,900
64	18,600	4100	3900	32,500	33,600	0	9100	14,411

Appendix II. Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate: Crude Antigen Preparations and Soluble Somatic Fractions

A. 7.5% polyacrylamide

1. Cytochrome C
2. Chymotrypsinogen A
3. Ovalbumin
4. Bovine serum albumin
5. Aldolase
6. Catalase
7. Crude male
8. Crude female

B. 12% polyacrylamide

1. Cytochrome C
2. Chymotrypsinogen A
3. Ovalbumin
4. Bovine serum albumin
5. Aldolase
6. Catalase
7. Crude microfilaria
8. Crude male
9. Crude female

C. 12% polyacrylamide

1. Female 2'
2. Female 3'
3. Female 4'
4. Female 5'
5. Female 6'
6. Female 7'
7. Female 8
8. Female 9'
9. Female 10'
10. Female 11'

D. 12% polyacrylamide

1. Female 12'
2. Female 13'
3. Female 14'
4. Male 1'
5. Male 2'
6. Male 3'
7. Male 4'
8. Male 5'
9. Male 6'
10. Male 7'

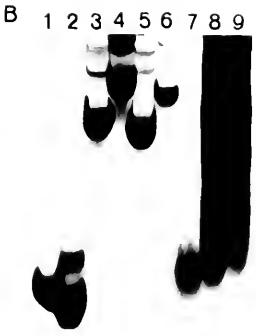
E. 12% polyacrylamide

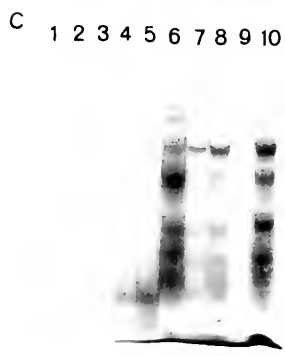
1. Male 8'
2. Male 9'
3. Male 10'
4. Male 11'
5. Microfilaria 1'
6. Microfilaria 2'
7. Microfilaria 3'
8. Microfilaria 4'
9. Microfilaria 5'
10. Microfilaria 6'

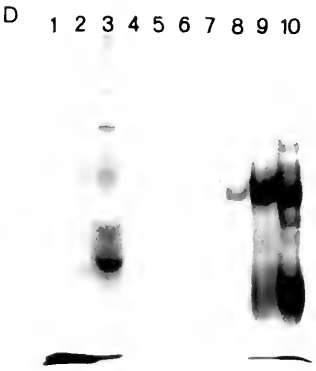
F. 10% polyacrylamide

1. Cytochrome C
2. Chymotrypsinogen A
3. Ovalbumin
4. Bovine Serum albumin
5. Aldolase
6. Catalase
7. Triton X-100 male
8. Triton X-100 female


A
1 2 3 4 5 6 7 8


B
1 2 3 4 5 6 7 8 9


C
1 2 3 4 5 6 7 8 9 10

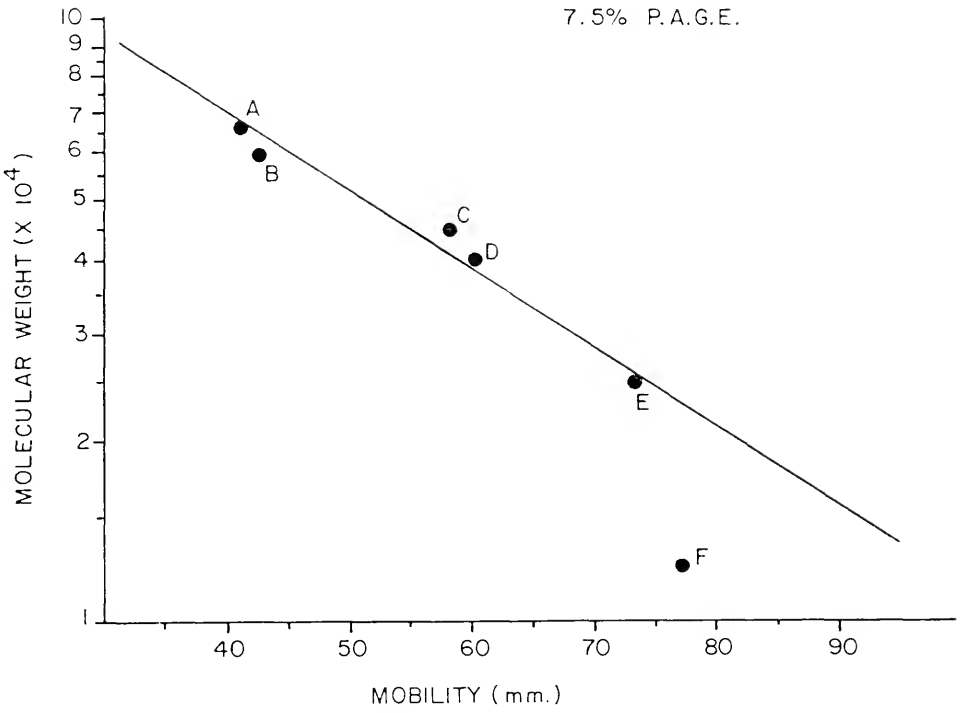

D
1 2 3 4 5 6 7 8 9 10


E
1 2 3 4 5 6 7 8 9 10


F
1 2 3 4 5 6 7 8

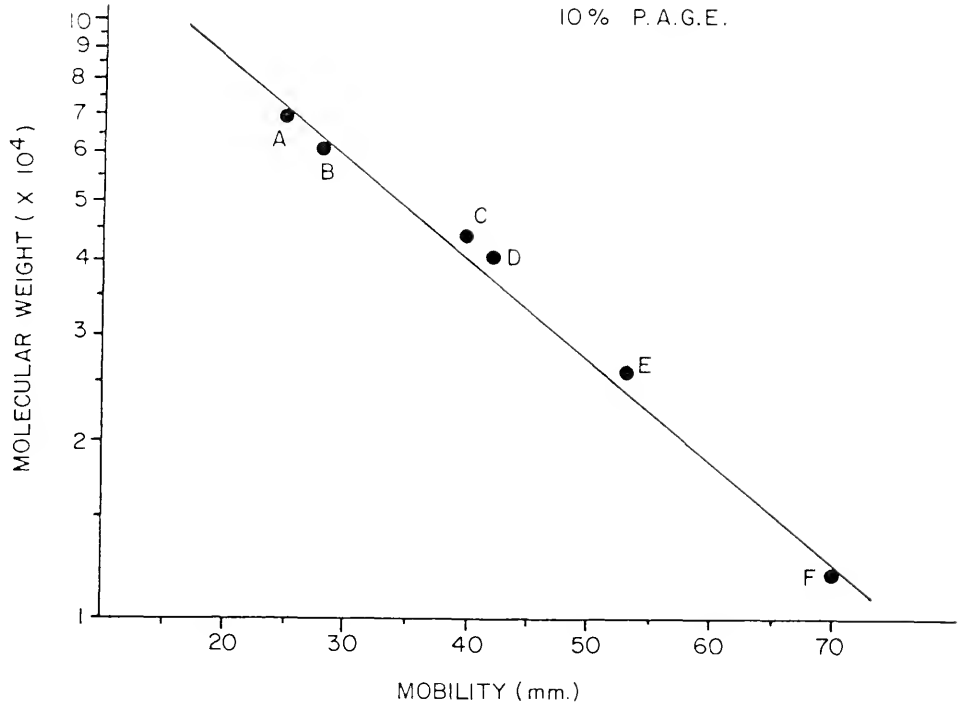
Appendix III. Standard Molecular Weight Curve
for 7.5% Polyacrylamide Gel

A= Bovine Serum Albumin
B= Catalase
C= Ovalbumin
D= Aldolase
E= Chymotrypsinogen A
F= Cytochrome C


7.5% P.A.G.E.
MOLECULAR WEIGHT (X 10^4)
10
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
40
50
60
70
80
90
MOBILITY (mm.)

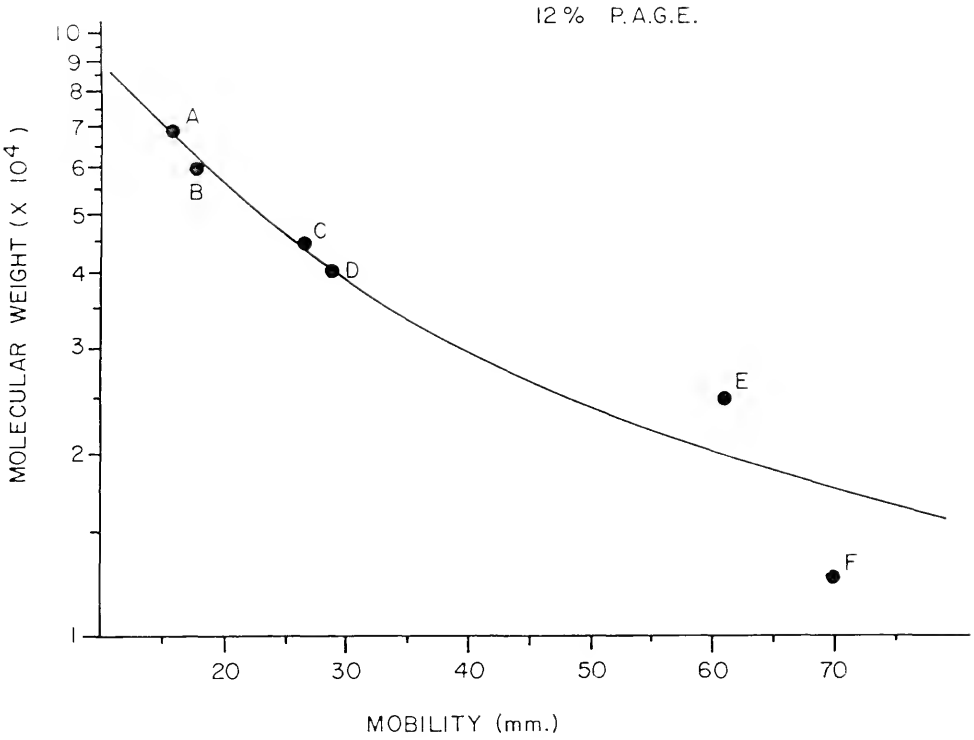
Appendix IV. Standard Molecular Weight Curve for 10% Polyacrylamide Gel

A= Bovine serum albumin
B= Catalase
C= Ovalbumin
D= Aldolase
E= Chymotrypsinogen A
F= Cytochrome C


10 % P.A.G.E.
MOLECULAR WEIGHT (X 10^4)
10
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
20
30
40
50
60
70
MOBILITY (mm.)

Appendix V. Standard Molecular Weight Curve
for 12% Polyacrylamide Gel

A= Bovine serum albumin
B= Catalase
C= Ovalbumin
D= Aldolase
E= Chymotrypsinogen A
F= Cytochrome C


12% P.A.G.E.
MOLECULAR WEIGHT (X 10^4)
10
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
20
30
40
50
60
70
MOBILITY (mm.)

Appendix VI. Polyacrylamide Gel Electrophoresis: Crude Antigen Preparations and Soluble Somatic Fractions

A. 1. Crude male

2. Crude female

3. Crude microfilaria

4. Male 1

5. Male 2

6. Male 3

B. 1. Male 4

2. Male 5

3. Male 6

4. Male 7

5. Female 1

6. Female 2

C. 1. Female 3

2. Female 4

3. Female 5

4. Female 6

5. Female 7

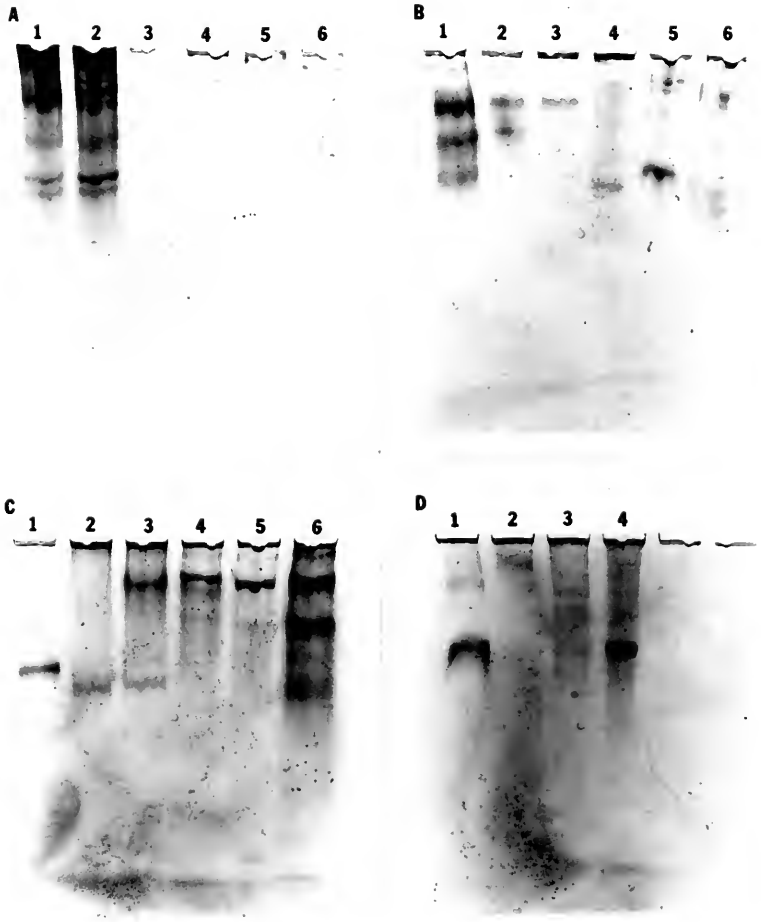
6. Microfilaria 1

D. 1. Microfilaria 2

2. Microfilaria 3

3. Triton X-100 male

4. Triton X-100 female


A
1
2
3
4
5
6
B
1
2
3
4
5
6
C
1
2
3
4
5
6
D
1
2
3
4

APPENDIX VII

Anti-*Dirofilaria immitis* Antibody Titers from All Dogs Over the Experimental Period

Weeks After Initial *D. immitis* Infection	Group A (noninfected)				Group B (single infection)				Group C (double infection)			
	H-3	K-5	K-3	H-4	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
-4	0[a]	0	1	-	0	1	0	1	1	1	0	1
-2	1	1	2	1	1	1	1	1	1	1	1	1
0	0	0	0	0	0	1	1	1	0	1	1	0
2	0	0	0	0	0	0	0	0	0	1	1	0
4	2	1	2	2	4	4	2	4	4	16	8	2
6	1	0	1	0	2	128	1	2	2	8	1	2
8	2	1	1	1	8	4	8	8	2	64	4	4
10	2	2	1	1	4	4	4	4	2	64	8	2
12	1	2	1	1	4	1	4	2	1	8	8	2
14	2	1	2	2	1	1	2	2	4	32	2	2
16	0	0	1	0	2	0	4	1	2	2	1	1
18	1	1	2	1	32	2	-	2	2	4	1	1
20	0	0	0	0	128	2	4	8	1	4	8	1
22	0	0	2	0	256	2	2	2	2	4	4	1

Anti-*D. immitis* Antibody Titers (Continued)

Weeks After Initial *D. immitis* Infection	Group A (noninfected)				Group B (single infection)				Group C (double infection)			
	H-3	K-5	K-3	H-4	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
24	0	1	0	1	128	1	4	2	1	4	1	1
26	1	0	0	1	8	1	1	2	2	16	2	2
28	1	2	1	1	64	2	8	2	2	4	2	2
30	2	1	0	1	256	1	32	2	4	4	2	1
32	0	-	0	1	1024	2	64	2	4	16	512	2
34	0	0	2	2	8	4	15	4	4	4	2	2
36	0	0	1	1	2	4	4	8	4	32	4	4
38	0	0	0	0	2	1	32	4	4	8	4	4
40	1	1	0	1	2	2	2	2	2	4	4	2
42	1	1	-	1	4	1	2	2	4	4	2	2
44	1	1	1	1	1	0	1	2	2	4	2	1
46	1	0	1	1	2	1	1	2	2	4	2	2
48	1	0	1	2	2	1	4	2	2	8	2	4
50	2	2	1	2	1	4	16	0	16	1024	4	256
52	0	2	0	2	4	1	2	4	4	128	128	64

Anti-*D. immitis* Antibody Titers (Continued)

Weeks After Initial *D. immitis* Infection	Group A (noninfected)				Group B (single infection)				Group C (double infection)			
	H-3	K-5	K-3	H-4	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
54	0	1	1	1	2	1	2	4	2	32	8	4
56	0	1	1	1	1	1	2	2	2	16	8	4
58	0	2	2	1	1	1	4	1	1	16	2	1
60	0	2	2	2	1	1	4	2	1	32	2	1
62	0	1	1	1	1	0	2	1	1	16	2	1
64	0	1	1	1	1	0	4	1	1	16	4	2

[a]Antibody titers are expressed as the reciprocal of the last dilution with hemagglutinating activity.

APPENDIX VIII

Mean Anti-Sheep Red Blood Cell Antibody Titers Before and After 2-Mercaptoethanol Treatment

Days After Primary Immunization	Group A (noninfected)				Groups B & C (infected)							
	H-3	K-5	K-3	H-4	K-4	H-6	K-1	H-5	H-7	K-2	K-6	H-2
0	0/0[a]	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	.5/.5	0/0
8	32/16	96/96	32/16	16/16	48/32	24/12	48/32	24/24	32/32	320/128	80/80	24/24
16	12/6	16/12	12/8	6/4	16/12	8/6	16/16	6/5	24/16	32/24	24/16	12/6
20	48/32	64/64	32/16	16/16	64/32	24/16	64/64	32/24	96/48	128/128	32/32	16/8
22	32/16	64/48	64/32	16/16	48/48	32/24	96/48	48/32	64/48	48/32	48/32	16/16
27	16/16	32/24	32/32	16/16	48/32	24/16	48/32	32/32	48/32	192/160	48/32	16/8
29	16/16	64/48	32/32	16/16	32/32	24/24	64/64	16/24	64/48	256/128	28/32	12/8

[a]Antibody titers are expressed as the mean of the reciprocal of the last dilution with hemagglutinating activity.

APPENDIX IX

Abbreviations Defined and Used in the Text.

B-cell - Thymus independent lymphocyte

Con A - Concanavalin A

cpm - counts per minute

DIA - *Dirofilaria immitis* antigen

EDTA - Ethylenediaminetetracetic acid

FCS - Fetal calf serum

FSSA - Female soluble somatic antigen

HAB - Hemagglutination buffer

HBSS - Hank's balanced salt solution

HRBC - Human red blood cells

IgG - Immunoglobulin class G

IgM - Immunoglobulin class M

IHA - Indirect hemagglutination

MFSSA - Microfilaria soluble somatic antigen

MSSA - Male soluble somatic antigen

PAGE - Polyacrylamide gel electrophoresis

PAGE-SDS - Polyacrylamide gel electrophoresis in sodium dodecyl sulfate

PAS - Periodic acid Schiff's stain

PBS - Phosphate buffered saline

PHA - Phytohemagglutinin P

PPD - Purified protein derivative-tuberculin antigen

PWM - Pokeweed mitogen

Abbreviations (Continued)

SDS - Sodium dodecyl sulfate

SK-SD - Streptokinase-streptodornase antigen

SRBC - Sheep red blood cells

T-cell - Thymus-dependent lymphocyte

TCA - Trichloroacetic acid

tris - 2-amino-2(hydroxymethyl)-1,3-propanediol

TSF - Triton X-100 solublized fractions of female cuticles

TSM - Triton X-100 solubilized fractions of male cuticles

2-ME - 2-mercaptoethanol

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BIOGRAPHICAL SKETCH

Robert Burton Grieve was born October 27, 1951, at Torrington, Wyoming. He graduated from Lingle High School, Lingle, Wyoming, in May, 1969. In September, 1969, Mr. Grieve initiated studies at the University of Wyoming, Laramie, where he received the Bachelor of Science degree in Microbiology in May, 1975. He worked as a diagnostic parasitologist at the Wyoming State Veterinary Laboratory from 1973 to 1974 while continuing graduate studies at the University of Wyoming. In May, 1975, he completed the Master of Science degree in Microbiology with the thesis in veterinary parasitology. Mr. Grieve enrolled at the University of Florida in September, 1975, to work toward the Doctor of Philosophy degree. He has held a graduate assistantship in the College of Veterinary Medicine (Institute of Food and Agricultural Sciences–Animal Research Facility) where he studied immunology and parasitology.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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